COMPOUNDS FOR METABOLISM STUDIES: RADIOACTIVE AROMATIC AMINES AND THEIR POSSIBLE DETOXICANTS

By

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I. INTRODUCTION

In 1856, an eighteen-year-old chemist, William Henry Perkin (76), oxidized an impure mixture of aniline and toluidine with sodium dichromate in an attempt to obtain quinine. His experiment failed to yield the compound he expected but, instead, gave him a black precipitate containing a small amout of a brilliant dye. Perkin, using rare vision and industry, commercialized his discovery and by 1857 mauveine was on the market. His new dye immediately was accepted by French dyers, thus introducing to the world the golden age of the synthetic aniline dye industry. Within a few years, millions of pounds of dyes and their intermediates were being made. Prosperity increased as the world began reaping the harvest from the organic chemical industry that synthetic dyes had initiated.

All too soon, however, the booming prosperity brought by the growing dye industry began to be marred. Goldblatt (38) states that in 1895 Rehn made the first report of the incidence of bladder tumors among workers in dye-stuff factories in Germany. Rehn's report did not receive the attention that should have been given to it. In investigations into the causes of neoplastic growth too little heed had been paid to the factual evidence of environmental cause. Man is most reluctant to connect health hazards with practices that are personally profitable or enjoyable.

He sought the cause of cancer in morphological, genetic, viral, or biochemical aspects of the disease.

Evidence of environmental cancer began to follow the dye industry wherever it spread. In various countries bladder cancer made its first appearance in association with the initiation of the dye industry (51). These bladder cancers were always associated with the actual manufacture of dyes and did not affect dyers who handled the finished product. Suspicion fell on the intermediates used in the manufacture of these dyes. A large percentage of the intermediates were aniline, alpha- and beta-naphthylamine, and benzidine--all belonging to the class of organic compounds known as aromatic amines.

These compounds were repeatedly tested for carcinogenicity on animals, but the results were always negative. Without experimental evidence to back up their suspicions, scientists were unable to induce manufacturers to adopt adequate methods of protection for their workers. Researchers were less reluctant to accept the aromatic amine as a carcinogen and in 1924 Kennaway (54) writing in the British Medical Journal accepted beta-naphthylamine as a cancer producing agent. Still there was no experimental evidence to support the suspicions. Repeated attempts to produce cancer by application of aromatic amines to rats and mice failed. The case against the dye intermediate seemed about to collapse completely for lack of evidence when Hueper et al. (52) used the dog as the experimental animal. This group of workers was able to report the production of bladder tumors in the dog by feeding beta-naphthylamine. Their success opened up a new field of research. Hueper's work was soon confirmed and the growing experimental and statistical evidence took on a new meaning to even the most skeptical.

A statistical study made by Müller (72) in Basle, Switzerland, where there are many aniline dye factories, revealed that cancer in the bladder was 33 times more frequent in workers in these factories than in the remainder of the male population. In one small manufacturing town in England, the mortality rate from bladder tumors among workers in a dye factory was thirty-eight times higher than among the insured male population of the town. It was reported from France that in one factory handling dye intermediates the incidence of vesical tumors among the workers was forty-three times that of the general population (38).

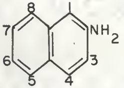
These accumulated statistics on the number of bladder tumors among industrial workers in factories using aromatic amines are startling. Coupled with experimental evidence these figures strongly support the belief that there is such a thing as "aromatic amine cancer." Apparently, dye manufacturers began to think so too, for they took much needed precautions to protect their workmen. Today in most dye companies, beta-naphthylamine per se is no longer used. The sulfonic acids of beta-naphthylamine, so important in the syntheses of colors, are now produced by sulfonation of beta-naphthol followed by amidation. This change in synthetic procedure represents a reduction in the hazard from one aromatic amine. Additional hazards still exist (81) and recent indictments of other intermediates (88, 6) point out the necessity for continued study of the physiological effects of aromatic amines.

A. Physiological Properties of Aromatic Amines

1. Effect of Chemical Structure

a) On Carcinogenicity

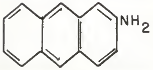
The production of bladder tumors in dogs from feeding beta-naphthylamine (52) stimulated research into the relationship of chemical structure to the carcinogenicity of aromatic amines. beta-Naphthylamine, I, is a relatively simple compound. Its basic structure consists of two fused benzene rings with an amino group in the 2-position. When the ring is numbered as shown in I, the beta- and the 2-position are synomynous. Apparently, the position of the amino group has carcinogenic significance, because



I beta-Naphthylamine

1-aminonaphthalene (alpha-naphthylamine) has not been shown to be carcinogenic (42) when tested without contamination from the 2-isomer.

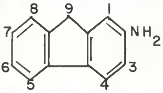
If the basic structure of 2-aminonaphthalene is enlarged to contain three rings as in 2-aminoanthracene, II,



II 2-Aminoanthracene

carcinogenicity is retained. Bielschowsky (13) has shown that several types of tumors may be produced in rats by using 2-aminoanthracene. Hence, increasing the size of the figure aromatic nucleus does not destroy the tumorigenic properties of aromatic amines. Here again, it should be noted that the 2-position has a particular importance. Bielschowsky has shown that if the amino group is in any other position the compound is inactive (13).

2-Aminofluorene, III, is also a tricyclic compound,



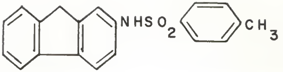
III 2-Aminofluorene

but in this amine the center ring is 5-membered. This compound as its acetylated derivative, 2-acetylaminofluorene (2-AAF) has been shown by many investigators (1, 4, 10, 69,

109) to produce a wide spectrum of tumors in a variety of species. The unexpected versatility of 2-AAF as a carcinogen led to an extensive and continuing research into the structural relationships of its cancer producing properties.

Bielschowsky (11) theorized that 2-aminofluorene (2-AF), the unacetylated compound, was the true carcinogen, and animal experiments have shown that 2-AF is about as carcinogenic as 2-AAF (68). In order to resolve the question of what chemical structure is the carcinogenic species, investigators called upon radioactive tracers to shed light upon the mechanism of activity of these compounds in the animal body. Weisburger and co-workers (100) employed in their studies 2-acetylaminofluorene-9-C-14 and 2-acetylaminofluorene-ω-C-14 prepared for them by Ray and Geiser (79). When 2-AAF-9-C-14 was administered by stomach tube, the radioactive carbon stayed with the aromatic nucleus showing that the ring system remained intact, but when 2-AAF-ω-C-14 was used, 40% was recovered as expired $C^{14}O_2$, revealing that the acetyl group was extensively hydrolyzed.

In order to demonstrate more conclusively that the free amine was necessary to carcinogenicity, Ray and Argus (77) studied the metabolism of a non-carcinogenic N-substituted derivative of 2-AF, the S-35-labeled 2-(p-toluenesulfonyl)-aminofluorene, IV. As was suspected by these

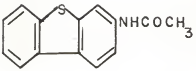


IV 2-(p-Toluenesulfonyl)-aminofluorene

investigators, the p-toluenesulfonyl group was not enzymatically hydrolyzed from the amine. It may be inferred from this information that conversion to the free amine is a necessary prerequisite for the carcinogenicity of a derivative of 2-AF. In further support of this hypothesis, Bielschowsky (14) reported that 2-methylaminofluorene and 2-dimethylaminofluorene, compounds that are partially converted to the amine, are less carcinogenic than 2-AAF.

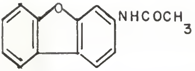
The first metabolite of 2-AF to be discovered was 7-hydroxy-2-aminofluorene (12) and it was hypothesized that the 7-position had to be free to hydroxylate in order for carcinogenic activity to be present. When chlorine or iodine was substituted in the 7-position to block hydroxylation, the compound showed no activity. When 7-hydroxy-2-aminofluorene was tested, however, it was found to be inactive or only slightly carcinogenic (47). When fluorine was put into the 7-position, activity was increased over that of 2-AAF (65). This information effectively exploded the hypothesis that the hydroxylation of the 7-position is a carcinogenic necessity.

Miller and associates have experimented with some changes in the methylene bridge of 2-AAF to investigate whether the aromatic nucleus of the compound must be kept intact to retain carcinogenicity (64, 66). Substitution of sulfur at the 9-position in place of the methylene bridge yields 3-acetylaminodibenzothiophene, V. The carcinogenicity



V 3-Acetylaminodibenzothiophene

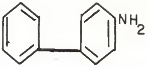
of this compound was not changed for mammary gland or ear duct tissue. If oxygen is substituted for the methylene bridge, 2-acetylaminodibenzofuran, VI, is obtained. This



VI 2-Acetylaminodibenzofuran

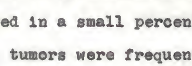
compound is somewhat less active toward mammary gland and ear duct tissue, but carcinogenic properties are retained. If an $\overset{O}{-S-}$ group is inserted for the methylene bridge, the carcinogenicity toward these two tissues is greatly lowered. Although tumors of some tissues can be produced by all three of these compounds, activity toward the liver is completely removed by alteration of the compound at the methylene bridge. The parent compound, 2-AAF, is an exceedingly potent liver carcinogen.

If the bridge of 2-AF is removed entirely, 4-aminobiphenyl, VII, is formed. This compound is carcinogenic in rats and some of its derivatives are more active than the parent compound (98).



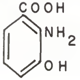
VII 4-Aminobiphenyl

If 4-aminobiphenyl is substituted with an amino group in the 4'-position, the common dye intermediate, benzidine, VIII, is formed. Once again the occurrence of liver tumors in animals is observed (88); hepatomas and adenocarcinomas of the rectum occurred in a small percentage of experimental animals and ear duct tumors were frequent. Bladder tumors which might be expected from a dye intermediate did not appear. However, when 3,3'-dihydroxybenzidine was tested on mice, bladder tumors were produced (6).



VIII Benzidine

The extensive alterations that are possible in the structure of aromatic amines without loss of neoplastic potency have made investigators wonder whether or not single ring amines could under some conditions be implicated. The single ring aromatic amine, aniline, has long been under suspicion as a contributor to bladder cancer in industrial dye factories (74) but researchers have never been able to show that it has cancer producing abilities in experimental animals. Recently, however, it has been suggested that aromatic amines with only one ring can be cancerigenic if a hydroxy group is ortho to the amine. Boyland and Watson (22) have investigated the activity of 3-hydroxyanthranilic acid, IX, by implanting it in pellets of paraffin wax and cholesterol in the bladders of mice. They reported the compound to be a carcinogen under these conditions. 3-Hydroxyanthranilic acid is a breakdown product of tryptophan and occurs naturally in the urine of man. The interesting theory of carcinogenicity and ortho-hydroxylation will be discussed later as it is related to species differences and protein binding.



IX 3-Hydroxyanthranilic acid

b) On Toxicity

Long years before the slow and insidious carcinogenic properties of some of the aromatic amines had been discovered, toxicity, a pronounced physiological characteristic of this class of chemical, had been studied with an eye toward the structural and metabolic relationships that may be involved.

The older literature often expresses toxicity as the "lethal dose" or the "minimum lethal dose," referring to doses which are fatal (LD) or the smallest dose which will kill a limited number of animals (MLD). Unfortunately, many instances are reported where the workers did not take into consideration the route of administration, the general health of the animal, or its age and weight. Duplication of such data is extremely difficult, and has brought about a trend in recent times to conduct experiments on a more controlled basis. This trend also involves the use of a larger number of animals, and the application of various statistical methods. Toxicity is now frequently expressed as that dose which will kill 50% of an experimental group (LD50) (86). In sub-lethal doses the symptoms of toxicity are usually described as they affect the blood picture or the nervous system.

The potent carcinogen, 2-acetylaminofluorene (2-AAF) was being tested for toxicity prior to its use as an insecticide when its carcinogenic properties were brought to light. In the toxicity tests, Wilson, DeEds, and Cox (109) found that 50 mg./kg. of body weight subcutaneously injected into rats produced no reaction of any kind that they could detect. They also gave male rats 2-AAF by stomach tube. Doses ranged from 50 to 1000 mg./kg. of body weight without any reactions being recorded. Although, under the conditions of the experiment, no acute toxicity was observed in rats, chronic symptoms were developed when 2-AAF was fed regularly in the diet. Rats given a one per cent diet of 2-AAF were dead within 22 days after the start of the experiment. All animals fed a 0.5 per cent diet were dead within 60 days, and those on a 0.25 per cent diet died within 93 days.

The structurally related carcinogenic 2-aminonaphthalene has a very low toxicity to man under the same industrial conditions that render it so treacherous to the dye worker. Hueper, Wiley, and Wolfe (52) investigated the toxicity of 2-aminonaphthalene in experimental animals while striving to produce tumors in dogs. Daily subcutaneous injections of four and five mg. for 113 days and oral administrations of 150 and 300 mg. for many months did not cause any distinct systemic effects. Doses of 500 mg./kg., however, will cause hematuria and after an hour, cyanosis, salivation, nausea, uneven walk, and methemoglobinemia, ending in death after several days have elapsed (35).

The toxicity of the non-carcinogen, 1-aminonaphthalene, was investigated before the turn of the century (95). It was found that in rabbits, the MLD was 300 mg./kg. of body weight. This dose caused tremors, muscular weakness, depression of the central nervous system, and occasionally death. The lethal dose (LD) was 400 mg./kg. Administration of 1-aminonaphthalene at this level was invariably fatal. It appears that the 1-isomer is more toxic than the 2-derivative; however, 1-aminonaphthalene does not have the destructive effect on the blood cells or the methemoglobinogenic action observed with some of the aromatic amines (99).

Benzidine, one of the aromatic amines credited with bladder tumors in dye workers, was investigated for toxicity (93). It was observed in dogs following the oral or subcutaneous administration of 200 mg./kg. of body weight that after a latent period of several hours, nausea, vomiting, and motor instability developed. Larger doses caused depression, dyspnea, clonic and tonic convulsions, and death. Benzidine, though more toxic than 2-aminonaphthalene, is less carcinogenic (50).

The simplest ring structure of the aromatic amine series is found in aniline, which has only one benzene ring attached to the amino group. In the human, serious poisoning effects result from as small a dose as 0.1 to 0.25 g. of this compound. Intoxication may occur from inhalation of aniline vapors, from swallowing the material, or from absorption through the skin (39).

Methemoglobin formation and subsequent loss of oxygen carrying capacity of the blood is the most noticeable characteristic of aniline poisoning. Cyanosis is so outstanding a feature that workers who are in constant contact with the chemical refer to aniline poisoning as "the blues." The symptoms of aniline poisoning in the human are vertigo, headache, mental confusion, and a desire to sleep.

In experimental animals, it has been shown that in addition to the effect on the blood, aniline has a direct effect on the nervous system. Rabbits, poor formers of methemoglobin, were used to show that after subcutaneous injection of one cc. of aniline/kg. of body weight, excitement and convulsions resulted followed by paralysis and death after 18 to 24 hours (91). Bonnet, Faye, and Mourot (16) showed in _in vitro_ experiments with nerve-muscle preparations, that aniline also paralyzes the peripheral nerve endings.

Acetylation of the amino group of aniline yields acetanilide, a compound which is also a methemoglobin former. However, introduction of the acetyl radical does slow down and lessen the cyanotic effect due to methemoglobin formation.

The actual formation of methemoglobin is probably due to phenylhydroxylamine formed _in vivo_ from ingested acetanilide or aniline (15).

Introduction of an hydroxy group para to the N-acetyl group of acetanilide with subsequent alkylation of the oxygen atom definitely lessens the toxicity of acetanilide, as for example in acetophenetidin. Both acetanilide and acetophenetidin are capable of yielding a chronic poisoning in man when they are taken over a long period for palliative purposes.

Lehmann (57) investigated the toxicity of mono-chloro-derivatives of some single ring aromatic amines. Using cats as his experimental animal he found that exposure daily for 21 days to vapors of 4-chloroaniline in a concentration of 0.068 mg./liter of air showed no toxic effects. However, subcutaneous injections of 125 mg./kg. proved to be a lethal dose (LD). The cats became cyanotic, nauseated, and developed twitchings and staggering gait which persisted for 60 hours when the animals died.

When Lehmann used 2-chloroaniline, exposure to vapor concentrations of 0.827 mg./liter for 10 hours/day for an eight day period showed no toxic effects in cats. Subcutaneous injection of 125 mg./kg. caused staggering gait and twitchings of the facial muscles, followed by recovery. More pronounced symptoms and death resulted from 310 mg./kg.

Experiments using 3-chloroaniline showed similar results to those obtained with 2-chloroaniline. Exposure to the vapors in a concentration of 0.3 mg./liter for 11 days showed no toxic effects. Subcutaneous injections of 125 mg./kg. caused some cyanosis and staggering gait but the animals recovered. When the dose was increased to 310 mg./kg. the animals died within seven hours.

These experiments indicate that the position of ring substitution affects the degree of toxicity, perhaps through the blocking of a position normally available to metabolism. 4-Chloroaniline is the most toxic of the three isomers tested in the cat. In this derivative, the p-position is blocked to hydroxylation and the formation of 4-aminophenol, which has been shown to be less toxic than aniline in several species (94).

No conclusions can be drawn from the toxic properties of a compound as to its degree of carcinogenic potency. Some of the most potent carcinogenic chemicals have a very low toxicity, and some highly toxic agents have not been shown to have neoplastic potency (50).

2. Species Differences

a) Reaction to Carcinogenicity

The delay in the incrimination of 2-aminonaphthalene as a carcinogen after it was first suspected to be the causative agent in bladder tumors was due to the failure to produce the same type of neoplasm in rats and mice. It was not until Hueper (52) used the dog that evidence against 2-aminonaphthalene was obtained experimentally. It was thus definitely demonstrated that species differ in their carcinogenic response.

Since Hueper's experiments, other workers have shown that species, strain, and sex are all important in determining the intensity of response as well as the location of induced tumors by chemical compounds in experimental animals.

Bonser *et al*. (17) has attempted to explain the species differences found for 2-aminonaphthalene by suggesting that 2-aminonaphthalene is not the direct carcinogen but, as may be the case with all chemicals capable of producing cancer, a metabolite may be the true culprit. The rat and the rabbit do not respond as readily to the neoplastic activity of 2-aminonaphthalene as does the dog. Bonser *et al*. (17), point out that there may be a relationship between the amount of administered 2-aminonaphthalene that is excreted as 2-amino-1-naphthol and its conjugates and the carcinogenic response in the animal. Implantation of pellets containing 2-amino-1-naphthol in the bladders of mice has established that this o-hydroxy derivative of 2-aminonaphthalene is carcinogenic when applied locally. The dog, susceptible to the neoplastic action of 2-aminonaphthalene, excretes 55-70% of an administered dose as 2-amino-1-naphthol. Less susceptible animals excrete smaller amounts of the o-hydroxy metabolite: the mouse, 20-40%; the rat, 12-15%; the rabbit, 5%. The main metabolite in these species is the acetylated 6-hydroxy derivative, 2-acetyl-amino-6-naphthol.

Differences in susceptibility of various animals and tissues to carcinogens are also well demonstrated by the action of 2-acetylaminofluorene (2-AAF) and its derivatives. The ability of 2-AAF to produce malignant growths was first shown by Wilson et al. in Slonaker strain rats (109). These workers incorporated the compound in a diet in a concentration of 0.031% or higher and fed for a period of three months. Irregular epithelial hyperplasia of the bladder, renal pelvis, liver, pancreas, and lungs resulted. Ten out of 39 animals developed bladder tumors. There were nine subcutaneous tumors, and three hepatomas and two leukemic infiltrations of the liver as well as occasional other types. On the other hand, Bielschowsky (10), using Wistar rats, found the greatest incidence of tumors in the liver, breast, and ear duct or zymol gland. In this strain there was an occasional tumor of the intestine, uterus, and skin but no changes whatsoever in the epithelial cells of the renal tract.

Using CBA strain mice, Armstrong and Bonser (4) further demonstrated that 2-AAF has a wide range of carcinogenic activity. They found that in this strain and species, the highest incidence of tumors was in the bladder. The results in CBA mice are closer to the results Wilson et al. obtained with their Slonaker strain of rats and differ from those obtained by Bielschowsky using the Wistar strain. The incidence of 2-AAF-induced bladder tumors in mice is higher in the male than in the female. This is in keeping with what is known of the disease in man.

Allison _et al._ used the dog as their experimental (1) animal for testing carcinogenicity of 2-AAF. In three out of six animals hepatic neoplasms developed. The urinary uric acid/allantoin ratio increased in these animals, reflecting the alteration in liver function. No bladder tumors were observed.

Bonser and Green (18) fed eight rabbits 2-AAF from one and one-half to five years. Urinary tract epithelial changes were observed ranging from simple hyperplasia and glandular metaplasia of transitional epithelium to infiltrating transition-cell and adenocarcinoma. A high incidence of ureteric tumors was noted. Neoplasms occuring in the uterus and ovary were regarded as spontaneous and independent.

Thus it is seen that with 2-AAF, the dog and Wistar rat develop a preponderance of liver tumors, but mice, rabbits, and Slonaker rats develop many tumors of the urinary tract and only a few in the liver.

The guinea pig has proven to be remarkably resistant to spontaneous or induced carcinogenesis. Breidenbach and Argus (26) attempted to induce tumors in this species by feeding 2-aminofluorene, (2-AF), in a stock diet. Two years after the initiation of the experiment, the surviving animals were sacrificed. No tumors were found. The data show that orally administered 2-AF while carcinogenic for the rat, is not for the guinea pig even though the guinea pigs received the chemical for a longer period of time, and in an amount equal to a 10-fold increase over that fed the rats. Making use of this information, the Weisburgers (101) applied the hypothesis concerning o-hydroxy metabolites of aromatic amines as the true carcinogens. Hydroxylation occurs in the rat at the 1-, 3-, 5-, 7-, and 8-positions of 2-AAF. In the guinea pig hydroxylation occurs at the 7- and 8-positions, with none to negligible amounts of hydroxylation at the 1-, 3-, and 5-positions of the ring system of 2-AAF. The 1- and 3-positions are ortho to the substituent on the ring and the 5-position is ortho to carbon-carbon linkage that joins the two phenyl rings of fluorene (101). Thus, the difference in the extent of o-hydroxylation of 2-AAF in the susceptible rat and the resistant guinea pig is analogous to the difference in amount of o-hydroxy metabolites of 2-aminonaphthalene excreted by animals susceptible and resistant to this compound (17).

Benzidine was shown by Spitz et al. (88) to be carcinogenic for Sherman strain rats. Oral and parenteral administration gave rise to sebaceous gland carcinomas in the glands adjacent to the auditory canal. Hepatomas and adenocarcinomas of the rectum occurred in a small percentage of the animals. Hepatic injury occurred early and consistently with subsequent cirrhosis similar in the late stages to portal cirrhosis of man. Bladder tumors did not occur. No tumors developed in rabbits or monkeys after administration of benzidine. One of seven dogs fed benzidine for five years had a papillary carcinoma of the urinary bladder, but whether this was a result of the feeding of the compound was not decided. No study has been made of the hydroxylation of benzidine in different species.

Although bladder tumors did not result from administration of benzidine, 3,3'-dihydroxybenzidine did yield bladder tumors when tested on mice. This o-hydroxy derivative of an important dye intermediate is found in the urine of men who have ingested benzidine (6).

b) Reaction to Toxicity

Although 2-aminonaphthalene has been definitely shown to display species differences in carcinogenic activity, very little study of its toxicity in different animals has been undertaken. To date toxicity experiments have been carried out primarily on the dog. Engel (35) found that doses of 500 mg./kg. caused hematuria in dogs, and after a latent period of an hour, cyanosis, salivation, nausea, vomiting, staggering gait, and methemoglobin, ending in death after several days had elapsed.

Several species have been tested for toxic reactions with the carcinogen 2-acetylaminofluorene (2-AAF). Wilson, DeEds, and Cox (109) have done acute toxicity studies with rats, mice, and rabbits. Male rats displayed no reactions of any kind with doses ranging from 50 to 1000 mg./kg. of body weight administered by stomach tube. Mice of the C57 strain, including both males and females, were injected subcutaneously with 2.5 mg. of 2-AAF without any toxic symptoms being recorded. Doses to rabbits ranged from 500 to 1000 mg./kg. of body weight without any indication of poisoning except a refusal to eat for several days.

2-AAF does not appear to be a former of methemoglobin as are compounds with simpler ring structures. Acetanilide, for example, forms a considerable amount in the animal body. The aminonaphthalenes are also moderate formers of methemoglobin (99). Possibly the fact that 2-AAF has not been shown to have acute toxicity is due to its failure to oxidize hemoglobin. The more dramatic property of carcinogenicity of 2-AAF has made the study of its toxicity less interesting to the investigator, and hence the subject has not been ardently pursued.

The toxicological picture of the non-carcinogenic analog of 2-AAF, acetanilide, varies to some degree with the different species and mode of administration. It was found that in dogs, 400 mg./kg. of body weight caused cyanosis, tremors, paresis, and weakness; larger doses caused anesthesia and decreased reactivity of the vagus nerve (92). In rabbits, 600-800 mg./kg. of body weight given orally had only a slight effect (45), but Smith and Hambourger found that 800 mg./kg. of body weight when administered by stomach tube to rats was fatal for 50% of the animals (LD50) (85). Smaller doses administered daily caused formation of methemoglobin in rats (84). Smith and Hambourger (85) give the minimum fatal dose (MLD) for mice by oral administration as being 800 mg./kg. of body weight.

Acetanilide does not produce methemoglobin *in vitro* (15). The observation that acetanilide is a former of methemoglobin *in vivo* must mean that some metabolite is responsible for methemoglobin formation. In 1913, Heubner (46) noted that the direct addition of the aminophenols to whole blood led to formation of methemoglobin with the order of effectiveness of the different isomers being: ortho, para, meta.

Different species vary in their ability to form methemoglobin and this is quite likely related to their methods of metabolizing acetanilide. The first stage in the metabolism of acetanilide is thought to be deacetylation to aniline (106), with subsequent hydroxylation and conjugation. In the dog, acetanilide is oxidized mainly to o-aminophenol. In rabbits the compound is deacetylated and converted into p-aminophenol followed by conjugation to an ethereal sulfate or a glucuronide. Acetanilide appears more toxic to dogs than to rabbits. o-Aminophenol is the main metabolite in dogs and is a more effective former of methemoglobin _in vitro_ (46) than is p-aminophenol, the main metabolite in rabbits.

It was repeatedly observed by the earlier investigators that different species of animals showed varying susceptibilities to methemoglobin formation by a given compound. Systematic studies made in later years (58, 87) have shown quantitative differences. Using as a criterion of sensitivity the reciprocal of the dose required to produce a stated concentration of methemoglobin, it was found that the cat was the most sensitive animal. If the cat is listed as 100, the range of other species for acetanilide is: man, 56; dog, 29; rat, 5; rabbit, 0; monkey, 0. For acetophenetidin, the following sensitivities were obtained: cat, 100; man, 63; dog, 35; rat, 5.

According to Spicer (87) 15 mg. of aniline/kg. of body weight produced 28.3% methemoglobin in the dog, 56.4% in the cat, and negligible amounts in the rat.

The variations in physiological responses from species to species point up the necessity of understanding metabolic pathways if one is to be able to gain the greatest use from animal experimentation. At the present time the beginning of a comparative study into metabolic pathways has been made. Much data are being accumulated to make it evident that the original structure of any physiological compound is not as important as the manner in which the organism is equipped to metabolize it. These metabolic pathways require further investigation.

B. Metabolic Reactions of Aromatic Amines

The term "Detoxication Mechanisms" as used in this thesis refers to any biochemical change that may occur in a foreign compound when administered in vivo. The change that takes place may not cause the compound to be less poisonous, for it is well known that many so-called detoxication mechanisms do not always "detoxicate" in the literal meaning of the word. For example, acetylation of sulfanilamide in the animal body causes a reduction in solubility with comcomitant crystallization of the compound and blockage of the kidney tubules. However, biochemists have come to accept "detoxication" as referring to the metabolism of a foreign compound in the animal body. A "foreign compound" is a pure chemical compound that does not naturally occur in the animal under consideration.

Eleven different methods are known by which foreign organic compounds may be metabolized by the vertabrate body, e.g., oxidation, reduction, and nine types of conjugation. Williams (105) lists these as the glucuronic acid conjugation, the ethereal sulfate conjugation, the hippuric acid synthesis or glycine conjugation, the cysteine conjugation or mercapturic acid synthesis, the ornithine conjugation or ornithuric acid synthesis, the glutamine conjugation, acetylation, methylation, and the thiocyanate detoxication. The detoxicative reactions of aromatic amines are limited to oxidation, acetylation, methylation, ethereal sulfate formation, and glucuronic acid synthesis. The specific type of mechanism which occurs depends upon the compound administered and the species to which it is administered. Some species cannot carry out certain detoxication mechanisms under any circumstances. For example, the rabbit cannot methylate and the dog cannot acetylate the aromatic amine (61).

In the opinion of Williams (108) the prediction as to the nature of the metabolites of a foreign organic compound must rest upon (a) analogies from the experience gained by studying closely related compounds and (b) knowledge concerning the degree of specificity of known (and unknown) enzyme systems of the body.

1. Oxidation

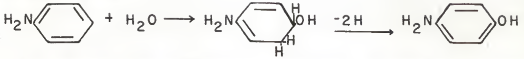
Oxidation is probably the most common metabolic change that takes place in foreign compounds administered to experimental animals. In the aromatic amine, it precedes conjugation with glucuronic and sulphuric acids by forming hydroxylated compounds.

The orientation of the hydroxyl groups that are introduced into the aromatic amine by living organisms has been the subject of much research. Out of the information that has accumulated it would appear that biochemical substitution of hydroxyl groups *in vivo* usually follows the same rules of substitution that apply in pure organic chemistry. That is, the amino group would be expected to direct hydroxylation to the o- or p-position of the ring (83) and this appears to be the case.

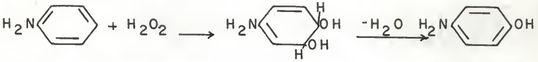
p-Hydroxylation of aromatic amines seems to be much more common than o-substitution. This could be partly attributed to steric hindrance in some species. For example, in the rabbit acetanilide is mainly hydroxylated in the p-position. The rabbit does not deacetylate acetanilide, but the dog which excretes much more of the o-hydroxylated metabolite of acetanilide rapidly deacetylates this compound. The fact that in the dog acetanilide undergoes deacetylation and o-oxidation whereas in the rabbit it undergoes primarily p-oxidation and no deacetylation, suggests that o-oxidation is hindered by stereo- or electro-chemical effects of the acetylamino group. The case of 2-aminonaphthalene also illustrates this effect. In the dog, 2-aminonaphthalene gives rise to 2-amino-1-naphthol and no acetylation of the amino group (104). Dobriner *et al*. (31) demonstrated in the rabbit that acetylation of 2-aminonaphthalene occurs and 2-acetylamino-6-naphthol is excreted. The hinderance of o-oxidation by acetylation of the amino group appears to be evident in this case.

The pathways by which these oxidations occur are not well understood, but several have been postulated.

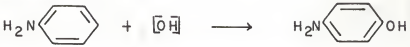
1) The addition of water to a double bond, followed by dehydrogenation:



2) Reaction with hydrogen peroxide to form a dihydrodiol intermediate followed by loss of water:



3) The direct substitution of a hydrogen atom by an hydroxyl group:



Udenfriend *et al.* (96) suggested that the large number of drugs and other foreign compounds which are hydroxylated in the body indicates that one or more non-specific hydroxlating mechanisms are present. This group has studied the role of ascorbic acid in the hydroxylation of compounds with aromatic character. *In vitro* studies with a system consisting of ascorbic acid, oxygen, and Fe^{++} yield compounds with phenolic properties. Evidence is presented to demonstrate that the hydroxyl group is directed to electronegative sites on the aromatic ring to yield products identical with those formed in the intact animal. Ascorbic acid is implicated in the hydroxylation of aromatic compounds *in vivo* as shown by experiments with the non-ascorbic acid producing guinea pig. When ascorbic acid was withheld from the animals, hydroxylation of acetanilide and aniline was greatly decreased, but when adequate ascorbic acid was supplied the animals were able to increase their rate of hydroxylation three-fold.

The exact nature of the oxidizing agent is not known. It has been shown that the active species is not ascorbic acid, dehydroascorbic acid, or a peroxide, but it is believed by Udenfriend (96) that the active agent may be an unknown oxidation product of ascorbic acid.

2. Conjugation

a) Acetylation

Acetylation of aromatic amines is accomplished by most species of animals tested. It had been asked (90) whether the acetylation of aromatic amines does not follow a different metabolic pathway from the mechanism involved in the acetylation of amino acids. The vast amount of research into biological acetylation is well summarized in the work of Lipmann (60) which seems to indicate that the mechanism of aromatic and aliphatic acetylation is the same. This leaves unexplained the inability of the dog to acetylate the aromatic amine, although this animal does acetylate the amino acid $-NH_2$ group.

Coenzyme A acts as the transfer agent for acetyl groups. Acetyl phosphate can react with reduced Co A to yield acetylated Co A and orthophosphate. Acetylated Co A then transfers the acetyl group to the receptor compound and reduced Co A is regenerated:

$$\text{Ac-P} + \text{CoASH} \rightleftarrows \text{CoASAc} + \text{P}$$

$$\text{CoASAc} + \text{R} \rightleftarrows \text{AcR} + \text{CoASH} \quad (89)$$

Energy for the transfer is derived from a high energy phosphate bond.

Generally, the liver is the site of acetylation, but other organs seem to participate in the reaction as shown by experiments with tissue slices and homogenates. Lipmann also demonstrated non-enzymatic acetylation of aniline with acetyl phosphate *in vitro*, but whether or not the reaction can be non-enzymatic *in vivo* is uncertain.

Acetylation is reversible with one enzyme catalyzing the reaction in either direction. Deacetylation of the aromatic acetylamino group varies with the species, however. It is extensive in the cat, dog, rat, and pigeon, but very small in man and rabbit. Not only does deacetylation vary with the species but also with the substituents on the ring. Bray and his associates (25) made *in vitro* studies on the percentage of hydrolysis of the acetyl group of acetanilide when the ring was substituted in the o-, m-, or p-positions. They found that when chlorine was in the o-position of acetanilide, hydrolysis was 96% complete; with m- substitution hydrolysis was 20%; and with p- substitution only 11% complete. Whether this relationship holds *in vivo* had not been previously determined before the present investigation.

It may well be that the deacetylation is a factor in the toxicity of aromatic amines. Certainly the free amines are more soluble in body fluids than are acetylated compounds. The type of detoxication of any given compound in any species governs the physiological response to the compound whether the response is toxic or carcinogenic.

b) Ethereal Sulfate Formation

The formation of the ethereal sulfate conjugate following hydroxylation is one of the detoxication mechanisms that lessens the toxicity of aromatic amines. The compound is rendered more soluble and, hence, more quickly excretable by the animal organism.

The biosynthesis of arylsulfates is at least a two stage process (9). The first reaction, which is rate-limiting, is the activation of sulfate ions by adenosine triphosphate (ATP) in the presence of an unknown co-factor. The activated sulfate then conjugates with the phenol under the control of an enzyme:

$$SO_4^+ + ATP \longrightarrow \text{active sulfate}$$

$$\text{phenol} + \text{active sulfate} \xrightarrow{\text{sulfatase}} \text{phenol sulfate}$$

There are four types of sulfatases so far discovered: phenolsulfatase, glucosulfatase, chondrosulfatase, and myrosulfatase. As is characteristic of enzymes, each is highly specific. For instance, taka-diastase, a plant phenolsulfatase, will hydrolyze only sulfuric esters with an aromatic radical directly attached through an ether linkage and will not attack esters with an aliphatic radical or any other type of sulfuric ester (37).

Sulfatases are present in most organs of higher animals with the liver, adrenals, and kidney being the most abundant sources of the enzyme. Evidence seems to indicate that different enzyme systems are involved in the synthesis and hydrolysis of sulfates (9) although the final decision on this subject must await the preparation of purer concentrates of the enzymes and the co-factors concerned.

c) Formation of Glucuronides

The formation of glucuronides provides a major pathway of detoxication *in vivo*. The manner of synthesis of glucuronic acid and subsequent conjugation with foreign and naturally occurring compounds has been given a place of importance in biochemical research. Tracer work with C-14 indicates that glucuronic acid is synthesized in the animal

$$ROC_6H_9O_6 + R'OH \xrightarrow{\text{beta-glucuronidase}} ROH + R'OC_6H_9O_6$$

body directly from glucose or an equivalent 6-carbon unit (32, 34, 70). These results are in contradiction to the earlier held belief that formation of glucuronic acid *in vivo* was through an unknown 3-carbon unit (107).

The second step in the synthesis of glucuronides, that is, the formation of the conjugate, may proceed by two pathways. One pathway depends on the participation of beta-glucuronidase as the synthesizing enzyme. The second route of glucuronide synthesis is via a transfer of the glucuronic acid moeity from uridinediphosphate glucuronic acid to a phenolic compound. This exchange is under the control of a transferase which is not beta-glucuronidase (36). The extent to which each synthesizing enzyme system operates in the organism is not known at present.

beta-Glucuronidase acting in its hydrolytic function may play an indirect role in the production of bladder cancer. This enzyme is found in the urine of man and its action frees the hydroxy amines from their glucuronides. As mentioned before, it has been postulated that the unconjugated o-hydroxy compound is necessary for carcinogenic activity of aromatic amines. If this is true, liberation of o-hydroxylated metabolites of ingested aromatic amines from their glucuronides, may cause a considerable accumulation of active carcinogens in the bladder. On the other hand, there is some evidence to indicate that the ethereal sulfates of o-hydroxy amines are not hydrolyzed by the sulfatases in the bladder (21).

3. Protein Binding

Miller and Miller (63) have shown that aminoazo dyes bind to the protein of rat livers when the animals are fed azo dyes. Since many of these azo compounds are carcinogenic Dyer and Morris (33) inquired into the possible protein binding of the carcinogenic aromatic amine, 2-acetylaminofluorene. Use was made of the heavy isotope of nitrogen, N-15 (3), to trace the presence of the carcinogen in the body tissues. Small but consistent excesses in the concentration of isotopic nitrogen were bound to the proteins of rat liver, but not in guinea pig liver, following administration of 2-acetylaminofluorene-N-15. As previously indicated the rat is extremely susceptible to the carcinogenic action of 2-AAF, but the guinea pig shows an amazing resistance to the chemical. In the rat, it was found also that 4-acetylaminofluorene, which is a non-carcinogen, did not bind with liver proteins.

Although it has been shown that non-carcinogens may in some instances bind to proteins (43, 66), on the basis of the collected evidence it has been suggested that protein binding is a necessary, but not sole, prerequisite to carcinogenesis (43). Just what change a carcinogen must undergo before protein binding can take place is, of course, a matter for speculation at the present time. Clayson (30) presented a working hypothesis which offers a basis for further research in the problem of the carcinogenicity of aromatic amines. He speculated that the difference in species response to the same aromatic amine was a reflection of the variation in metabolism from species to species. For example, in its quantitative aspects, the conversion of 2-aminonaphthalene to 2-amino-1-naphthol derivatives offers an explanation of the differences in species susceptibility to 2-aminonaphthalene carcinogenesis.

Clayson's suggestions are 1) that amines which contain an o-hydroxy group in an aromatic system of two or more rings may be carcinogenic either in their own right or as a result of their further reaction and 2) the reason why some aromatic amines induce tumors whereas others do not is that some amines are more readily converted in the body to o-hydroxy amines than are others.

In the case of 2-acetylaminofluorene which is carcinogenic in the rat and non-carcinogenic in the guinea pig, it has been noted that the resistant animal does not hydroxylate the carcinogen in the 1-, 3-, or 5-positions which are the ortho positions. The rat, which is susceptible to the carcinogenic action of 2-acetylaminofluorene does metabolize the compound by hydroxylation in these ortho positions (101).

Gutmann and Peters (41) have executed an interesting experiment on the deacetylation, hydroxylation, and protein binding of 2-AAF with liver slices and liver homogenates.

Their work has shown that the liver is a site of both deacetylation and hydroxylation. However, while deacetylation is accomplished by both slices and homogenates, only slices hydroxylate the carcinogen.

When tissue slices were incubated with 2-acetylaminofluorene labeled with C-14 in the 9-position, acetone failed to extract the radioactive material and precipitation of the protein by trichloroacetic acid failed to remove all the radioactivity from the proteins, thus, protein binding had been effected. However, when the tissues were homogenized, protein binding did not take place as shown by the absence of radioactivity. Gutmann and Peters, therefore, believed that either the intact cell is required for binding, or the bound compound may be an hydroxy metabolite which is produced by slices, but not by homogenates. A third possibility advanced is that both the intact cell and hydroxy metabolite are necessary for protein binding.

II. THE PROBLEM

A. The Rationale

The physiological importance of aromatic amines has been established in the Introduction. Possible relationships between the detoxication mechanisms and physiological response of an experimental animal have been suggested but further work is necessary to understand some of the factors concerned.

The present investigation was designed to establish relationships between the physiological activity of an aromatic amine and the type and extent of detoxication that the compound undergoes *in vivo*.

Acetanilide has analgesic and antipyretic properties and also exerts a toxic effect. This compound detoxifies by deacetylation and hydroxylation followed by conjugation with glucuronic acid or sulfate ions. *In vitro* studies indicate (25) that substitution of the various positions of acetanilide affects its deacetylation markedly. Such substitution would be expected to affect the position and degree of hydroxylation; a difference in hydroxylation might in turn affect the physiological properties of the compounds. The substituent chosen was chlorine so that the radioactive form, Chlorine-36, could be used as a tracer as well as the blocking group. 2- and 4-Chloroacetanilide-Cl-36 had been previously prepared by the author (73) and the distribution of these compounds was studied in the rat and guinea pig (40, 78).

During these investigations it was noted that 100 mg./kg. of body weight of 4-chloroacetanilide injected intraperitoneally into rats caused the animals to become cyanotic and prostrate; injection of the same dosage of 2-chloroacetanilide did not appear to adversely affect the animals. This apparent difference of toxicity was reflected in the rate of excretion of the two isomers in rats and guinea pigs. In both species the 2-chloroacetanilide-Cl-36 was eliminated very rapidly with most of the dose being excreted in the urine within six hours. Maximum excretion of 4-chloroacetanilide was at twenty-four hours. This was the information that suggested that the monochloro ring-substituted derivatives of acetanilide would be of value in an investigation into the relationship between the physiological activity and detoxication of a compound.

The present investigation was a study of the relationship of metabolism to the relative toxicity of 2-, 3-, and 4-chloroacetanilide in the rat. In order to accomplish this, it was necessary to synthesize the third isomer, 3-chloroacetanilide, with the radioactive label, Chlorine-36. The other two monochloroacetanilides labeled with Chlorine-36 had been previously prepared.

It was also necessary to synthesize the postulated hydroxy metabolites of 2-, 3-, and 4-chloroacetanilide for the purposes of identification.

The relative toxicity of the three isomeric chloroacetanilides was established in the mouse and rat, and the detoxication of each compound was followed through qualitative and quantitative recovery of the metabolites in the urine of rats injected intraperitoneally with 2-, 3-, or 4-chloroacetanilide-Cl-36.

B. Methods

1. Preparation of Compounds

a) 3-Chloroacetanilide-Cl-36

The first step in the research was to prepare the third ring-substituted chloro-isomer, 3-chloroacetanilide, labeled with Chlorine 36. In any radioactive synthesis, the important consideration is to obtain a satisfactory yield based on the radioactive element. The reaction normally chosen to synthesize 3-chloroacetanilide would probably be the Sandmeyer reaction. However, this reaction requires a large excess of chloride ions (80) that would dilute the Chlorine-36. A synthesis that gave better yields based on chlorine had, therefore, to be found.

In the attempted preparation of 3-chloroacetanilide by the direct chlorination of nitrobenzene a catalyst was required. This catalyst, however, combined with and rendered unavailable the radioactive chloride ions. Another synthesis involving replacement of the carboxyl group of the silver salt of 3-nitrobenzoic acid by chlorine was also attempted unsuccessfully. Chlorination of aniline in sulfuric acid to utilize the meta-directing characteristics of the $-NH_3^+$

group yielded negligible returns.

3-Chloroacetanilide was successfully prepared in good yield based on chlorine through the chlorination of 3-nitrobenzenemercuric acetate. The 3-chloronitrobenzene thus formed was readily reduced and acetylated (Fig. 1).

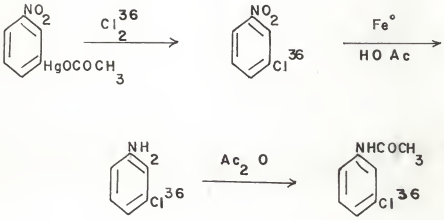


Figure 1

b) Metabolites of 4-Chloroacetanilide (Fig. 2)

The urinary metabolites that might be expected from injecting 4-chloroacetanilide into the living animal would probably be dependent upon the directing influence of the substituents on the ring. Both the N-acetyl and the chloro group are o-p directors, although the N-acetyl group is more efficient than the halogen as a director. The free amino group is a still more powerful o-p director. Therefore, hydroxylation should occur at the 2-position, whether the

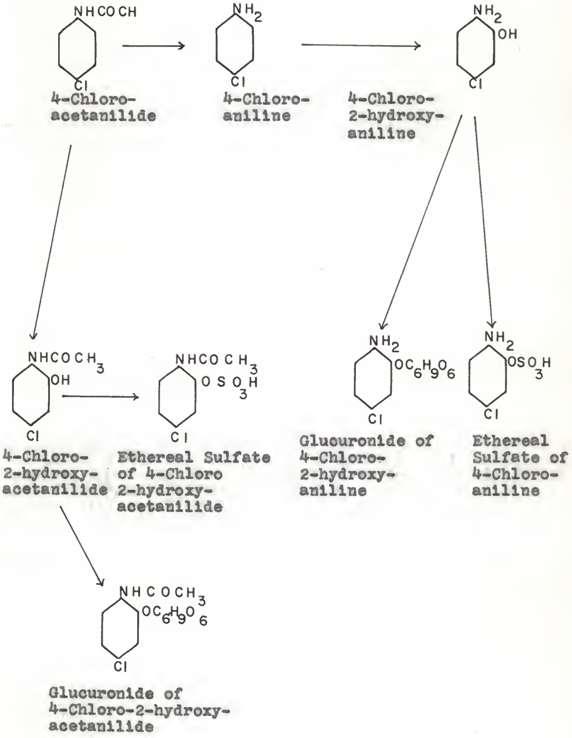

NHCOCH
Cl
4-Chloro-
acetanilide
NH2
Cl
4-Chloro-
aniline
4-Chloro-
2-hydroxy-
aniline
NH2
OH
Cl
NHCOCH3
OH
Cl
4-Chloro-
2-hydroxy-
acetanilide
NHCOCH3
OSO3H
Cl
Ethereal Sulfate
of 4-Chloro
2-hydroxy-
acetanilide
NH2
OC6H9O6
Cl
Glucuronide of
4-Chloro-
2-hydroxy-
aniline
NH2
OSO3H
Cl
Ethereal
Sulfate of
4-Chloro-
aniline
NHCOCH3
OC6H9O6
Cl
Glucuronide of
4-Chloro-2-hydroxy-
acetanilide

Figure 2

compound is deacetylated or not. The metabolites that would be expected to be formed from 4-chloroacetanilide are: 4-chloroaniline; 4-chloro-2-hydroxyacetanilide and its glucuronide and ethereal sulfate; 4-chloro-2-hydroxyaniline and its glucuronide and ethereal sulfate.

4-Chloroacetanilide and the deacetylated compound, 4-chloroaniline, have been known for many years (44). These are made commercially and were purchased from the Eastman Kodak Company and recrystallized to a state of high purity from organic solvents.

4-Chloro-2-hydroxyaniline and 4-chloro-2-hydroxyacetanilide were prepared by Hodgson and Kershaw in 1928 (48) and their procedures were followed with minor modifications. 3-Chlorophenol was nitrated to yield two isomers (49), 2-chloro-4-hydroxy- and 4-chloro-2-hydroxynitrobenzene. 4-Chloro-2-hydroxynitrobenzene was reduced with sodium hyposulfite to yield 4-chloro-2-hydroxyaniline. This compound was then acetylated to yield 4-chloro-2-hydroxyacetanilide (Fig. 3).

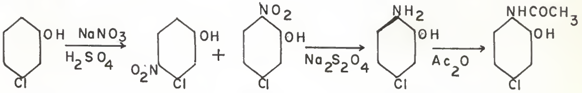


Figure 3

The sulfate esters and glucuronides were identified as the unconjugated compounds after hydrolysis of separate aliquots of radioactive urine by phenolsulfatase (taka-diastase) and beta-glucuronidase.

c) Metabolites of 3-Chloroacetanilide

The o-p directing influence of the chloro and N-acetyl or amino groups are working in unison in 3-chloroacetanilide. Hydroxylation of the compound, therefore, would be expected in the 4- and 6-positions of the molecule. The expected metabolites are: 3-chloroaniline, 3-chloro-4-hydroxyaniline and 3-chloro-4-hydroxyacetanilide, 3-chloro-6-hydroxyaniline, and 3-chloro-6-hydroxyacetanilide. These hydroxy compounds would probably appear unconjugated and as the ethereal sulfates and glucuronides (Fig. 4).

3-Chloroacetanilide and 3-chloroaniline were purchased from Eastman and purified by recrystallization and distillation respectively. The chloroaminophenols were prepared by reduction of commercially obtained 2-chloro-4- and 6- nitrophenol. Subsequent acetylation of the amino group with acetic anhydride yielded the N-acetyl derivatives. The presence of sulfates and glucuronides was established by hydrolysis of separate aliquots of radioactive urine by phenolsulfatase and beta-glucuronidase with subsequent isolation of the unconjugated phenols.

d) Metabolites of 2-Chloroacetanilide

In 2-chloroacetanilide the o-p directing influence of the amino or the N-acetyl groups are opposed to the directing

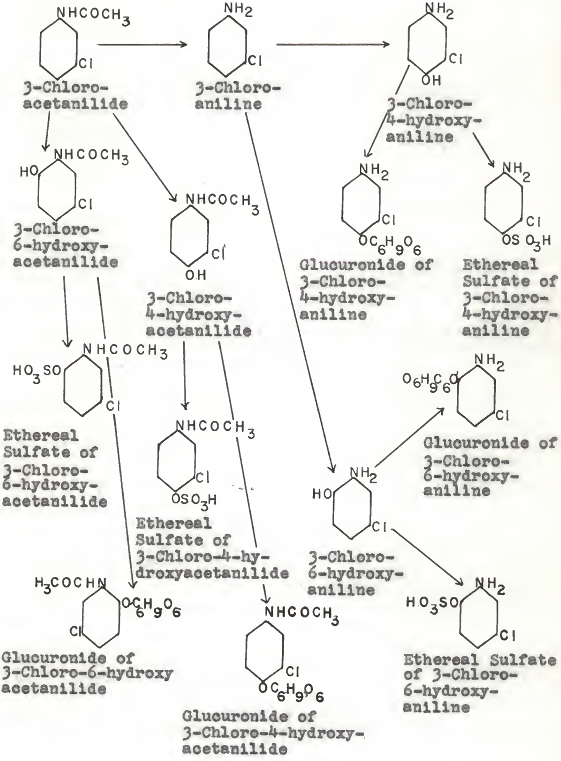

NHCOCH3
Cl
3-Chloro-acetanilide
NH2
Cl
3-Chloro-aniline
NH2
Cl
OH
3-Chloro-4-hydroxy-aniline
NHCOCH3
HO
Cl
3-Chloro-6-hydroxy-acetanilide
NHCOCH3
Cl
OH
3-Chloro-4-hydroxy-acetanilide
NH2
Cl
OC6H9O6
Glucuronide of 3-Chloro-4-hydroxy-aniline
NH2
Cl
OSO3H
Ethereal Sulfate of 3-Chloro-4-hydroxy-aniline
NHCOCH3
HO3SO
Cl
Ethereal Sulfate of 3-Chloro-6-hydroxy-acetanilide
NHCOCH3
Cl
OSO3H
Ethereal Sulfate of 3-Chloro-4-hydroxyacetanilide
O6H9C6O
NH2
Cl
Glucuronide of 3-Chloro-6-hydroxy-aniline
NH2
HO
Cl
3-Chloro-6-hydroxy-aniline
H3COCHN
OC6H9O6
Cl
Glucuronide of 3-Chloro-6-hydroxy acetanilide
NHCOCH3
Cl
OC6H9O6
Glucuronide of 3-Chloro-4-hydroxy-acetanilide
NH2
HO3SO
Cl
Ethereal Sulfate of 3-Chloro-6-hydroxy-aniline

Figure 4

influence of the chloro group. It would be expected, however, that hydroxylation would be in the positions controlled by the more efficient director which would be the N-acetyl or the free amino group. If, then, the orientation follows the rules of organic chemistry, 4- and 6-hydroxy compounds would be expected to be formed *in vivo*. The expected metabolites are, therefore: 2-chloroaniline; 2-chloro-4-hydroxyacetanilide, unconjugated and as the glucuronide and the ethereal sulfate; 2-chloro-4-hydroxyaniline, unconjugated and as the glucuronide and ethereal sulfate; 2-chloro-6-hydroxyacetanilide, unconjugated and as the glucuronide and ethereal sulfate; 2-chloro-6-hydroxyaniline, unconjugated and as the glucuronide and ethereal sulfate (Fig. 5).

2-Chloroaniline and 2-chloroacetanilide were purchased from Eastman Chemical Company and recrystallized from suitable organic solvents. 2-Chloro-4-hydroxyaniline was prepared by the method of Hodgson and Kershaw (48) through the reduction of 2-chloro-4-hydroxynitrobenzene which was obtained as a companion product in the preparation of 4-chloro-2-hydroxynitrobenzene from the nitration of 3-chlorophenol (47), [see II, B, 1, , Fig. 3]. Acetylation of 2-chloro-4-hydroxyaniline in benzene yielded 2-chloro-4-hydroxyacetanilide.

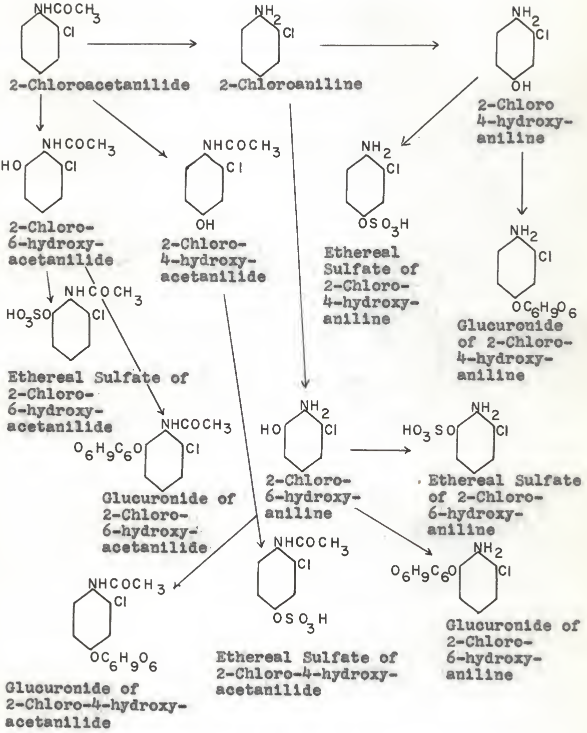

2-Chloroacetanilide
2-Chloroaniline
2-Chloro 4-hydroxyaniline
2-Chloro-6-hydroxyacetanilide
2-Chloro-4-hydroxyacetanilide
Ethereal Sulfate of 2-Chloro-4-hydroxyaniline
Glucuronide of 2-Chloro-4-hydroxyaniline
Ethereal Sulfate of 2-Chloro-6-hydroxyacetanilide
Glucuronide of 2-Chloro-6-hydroxyacetanilide
2-Chloro-6-hydroxyaniline
Ethereal Sulfate of 2-Chloro-6-hydroxyaniline
Glucuronide of 2-Chloro-6-hydroxyaniline
Glucuronide of 2-Chloro-4-hydroxyacetanilide
Ethereal Sulfate of 2-Chloro-4-hydroxyacetanilide
NHCOCH3
NH2
Cl
OH
HO
HO3SO
OSO3H
OC6H9O6
O6H9C6O

Figure 5

2-Chloro-6-hydroxyaniline was prepared by the reduction of 2-chloro-6-hydroxynitrobenzene. This nitro compound was prepared by nitration of 3-chlorophenol as described by Hodgson and Moore (49). 3-Chlorophenol was disulfonated to block the vulnerable 4- and 6-positions. Nitration was thus forced to the 2-position. Hydrolysis of the sulfonic acid groups by superheated steam yielded 2-chloro-6-hydroxynitrobenzene. 2-Chloro-6-hydroxynitrobenzene was reduced with sodium hyposulfite to yield 2-chloro-6-hydroxyaniline. Acetylation of this amine in water yielded 2-chloro-6-hydroxyacetanilide (Fig. 6).

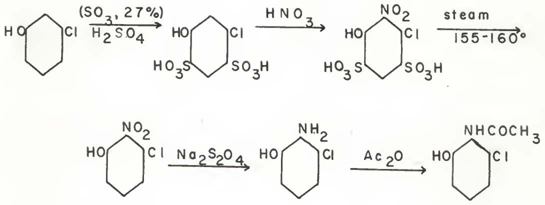


Figure 6

2. Determination of Presence of Metabolites

Inverse isotope dilution was the method chosen to identify detoxication products in the urine of the experimental animals. This method depends upon the use of radioactive tracers. If the administered drug is labeled with a radioactive element in a position where it is not readily removed by detoxication processes, then the metabolites of that drug excreted in the urine will carry the radioactive tag by which identification can be made.

The inverse isotope dilution technique is based on the addition of a quantitatively weighed amount of a known non-radioactive carrier to a solution containing radioactive material. Any compound in the radioactive solution that is chemically identical with the carrier will be inseparable from the carrier if the two are brought into equilibrium with each other. Careful isolation and subsequent purification will permit quantitative estimation to be made of the amount of radioactive compound present (28). That is, if a known amount of a non-radioactive compound, X, is added to a radioactive mixture containing some labeled X, the two, being chemically identical, become inseparable and the specific activity in counts per minute per milligram of the compound will be due to the mixture of labeled and unlabeled X. If the amount of unlabeled X is much larger than the amount of labeled X, then, the weight of the labeled material can be disregarded. The percentage of X in the mixture can be calculated by multiplying the specific activity by the amount of X added to the mixture, dividing the product by the total radioactivity of the solution and multiplying by 100 (102).

Use of the method of inverse isotope dilution permits accurate analysis of microgram concentrations or less. Sufficient unlabeled material may be added to insure ease of isolation and purification of the material after mixing. The only limitation on the amount of unlabeled material which may be added is the original isotopic concentration of the labeled material. Obviously, the material must be active enough to undergo the dilution of the experiment and still have measurable activity.

The great advantage of the inverse dilution technique that dictated the choice of the method for this experiment is that the material added in the dilution step did not need to be labeled. Quantitative isolation was not necessary at any stage of the analysis; it was only necessary to isolate a pure sample in a measurable quantity which could then be subjected to isotopic analysis.

The disadvantage of the method is the need of great purity of the final compound (111). It must always be kept in mind that a very small contamination of radioactivity would cause the recorded results to be in large error. Extreme care had to be taken on this point since more than one metabolite in the urine was labeled with the same isotope, Chlorine-36. Several methods were used to obtain this purity. One was recrystallization from different solvents to constant isotopic concentration (111). Another method used was to recrystallize from one solvent until the recrystallizing solution maintained constant activity (110). A combination of the two methods was also profitably employed.

3. Toxicity Studies with Isomeric Chloroacetanilides

Relative toxicity of the chloroacetanilides was determined in both the mouse and the rat. In order for a toxicity study to have value, a fairly large number of animals has to be used in the experiment. For economic reasons, the mouse is more practical when several compounds are to be tested. The first and more elaborate toxicity studies, therefore, were carried out in the mouse, using groups of 10 animals per dose. Confirmation studies were then made in the rat using groups of four animals. Because of the small size of the groups the study on the rats does not have great statistical value if taken alone, but it shows the relative toxicities of the compounds in the species used for the metabolism study. Details of the determination of the relative toxicities of the compounds are given in section III, C.

III. EXPERIMENTAL DETAILS

A. Preparation of Compounds

1. 3-Chloroacetanilide-Cl-36

a) Attempted Preparations of 3-Chloroacetanilide

(1) Chlorination of the silver salt of 3-nitrobenzoic acid.--The silver salt of 3-nitrobenzoic acid was prepared by the method of Zingaro *et al.* (112). One hundredth mole (2.74 g.) of this silver salt was suspended in 60 ml. acetic acid, and 0.04 mole of chlorine gas was generated and passed into the suspension at room temperature. The suspension became very thick as AgCl was formed. 3-Nitrobenzoic acid was also liberated but the expected product, 3-chloronitrobenzene, was not recovered. The experiment was repeated at reflux temperature, but again no expected product was recovered although CO_2 was caught in a $Ba(OH)_2$ trap, indicating that the carboxylic acid group was released from the benzene ring.

(2) Direct chlorination of nitrobenzene.--Two and four tenths grams (0.04 mol) nitrobenzene was dissolved in 20 ml. acetic acid and aluminum-amalgam catalyst added. Four hundredth mole of chlorine gas was generated and passed into the reaction mixture. The catalyst disintegrated and the aluminum apparently was bound with the chlorine, but no product was formed. The procedure was repeated using iodine as a catalyst (56) but no product was recovered. The reaction was repeated over a range of temperatures from 0° to 100°

using iodine or anhydrous $FeCl_3$ as catalysts but the amount of product recovered was negligible. Evidently a larger excess of chlorine than is practical for the radioactive synthesis is necessary.

(3) Chlorination of aniline in sulfuric acid.--The directing influence of the $-NH_3^+$ group is to the meta position so it was hoped that 3-chloroaniline might result from chlorination of aniline in a strong acid. Two ml. (0.02 mol) of aniline was dissolved in excess concentrated sulfuric acid. Chlorine gas (0.04 mol) was generated and passed into the solution at 0°. The solution was neutralized very slowly with NH_4OH and steam distilled. A yellowish oil was extracted in benzene and acetylated with acetic anhydride at 60°. The only product isolated was impure acetanilide. There was no chlorine in the molecule. This procedure was repeated at temperatures ranging from 0° to 190°. A trace of chlorine was found in the product when the reaction was carried out at room temperature, but the yield was only 0.67%. Again, apparently unlimited chlorine gas is necessary.

b) Preparation of 3-Chloroacetanilide-Cl-36

(1) Sodium salt of 3-nitrobenzenesulfinic acid (59).--Twenty-two grams of 3-nitrobenzenesulfonyl chloride (Eastman Kodak Company) was dissolved in a concentrated solution of sodium sulfite. After complete dissolution on the steam bath with periodic additions of NaOH to maintain alkalinity,

the solution was filtered and evaporated. Boiling ethanol extracted the yellow, crystalline, non-melting sodium salt of 3-nitrobenzenesulfinic acid. Yield; 13.2 g., 63%.

(2) 3-Nitrobenzenemercuric acetate.--3-Nitrobenzenemercuric acetate was prepared from the sodium salt of 3-nitrosulfinic acid by a modification of Kharasch and Chalkley's (55) preparation of 3-nitrobenzenemercuric chloride. Ten grams of 3-nitrosulfinic acid (sodium salt) dissolved in 50 ml. of water was added to 30 g. mercuric acetate in 150 ml. 95% ethanol. Three and two-tenths ml. acetic acid was added. The mixture was refluxed on the steam bath for 16 hours, filtered hot and the filtrate cooled slowly. 3-Nitrobenzenemercuric acetate precipitated out in white crystalline sheets. Yield; 18.3 g., 82.5%. M. p. 170°. Recrystallization of the crude product from 95% ethanol yielded a lustrous, plate-like crystalline compound, m. p. 173°.

(3) 3-Chloronitrobenzene-Cl-36.-- Five and one-tenth grams (0.0135 mol) of 3-nitrobenzenemercuric acetate, m. p. 173°, dissolved in 25 ml. of glacial acetic acid kept at 80-90° was slowly gassed with Cl_2^{36} (0.027 mol) in the following manner. Two generators were set up in series to generate chlorine gas. The first generator contained 200 microcuries HCl-36 which had been received in this form from Oak Ridge National Laboratories on allocation from the Atomic Energy

Commission. One hundred microcuries was contained in 2.0 ml. 6 N HCl-36 (0.012 mol). The second hundred microcuries was contained in 7.8 ml. 2.48 N HCl-36 (0.019 mol). This acid was oxidized to Cl_2^{36} with 4.74 g. $KMnO_4$ (0.03 mol) and 3.3 ml. conc. H_2SO_4 (0.06 mol). The gas was conducted through a drying tube containing concentrated H_2SO_4 and into the solution of 3-nitrobenzenemercuric acetate. A gas trap containing saturated KOH was connected to the reaction flask. This trap was in turn connected to an elevated water bottle used to apply steady and gentle suction to the gas train. The Cl_2^{36} generator was swept out with inactive chlorine gas from a second generator in which 3.8 ml. 6 N HCl (0.023 mol) was oxidized to Cl_2 with 3.63 g. $KMnO_4$ (0.023 mol) and 2.6 ml. conc. H_2SO_4 (0.046 mol). The entire system was then swept out with CO_2. After the train was completely swept free of chlorine gas, the reaction tube was corked and set in the cold room at 5° for 12 hours. The solution was poured into a 500 ml. round-bottomed flask, and made neutral with 20% NaOH. The yellow oil that appeared in the neutral liquid was steam distilled. Crystalline 3-chloronitrobenzene, m. p. 45° (56) was collected.

(4) 3-Chloroaniline-Cl-36.--The receiving flask from the distillation of 3-chloronitrobenzene was used as a reduction vessel. This flask was fitted with a stirrer and reflux condenser. Forty ml. 60% acetic acid and two grams

of powdered iron in two portions were added. The first was added at the beginning of the reaction, and the second, 45 minutes after the reduction had started. The total reflux time of the reaction was five hours. The solution was cooled, made alkaline with saturated NaOH, and steam distilled. 3-Chloroaniline-Cl-36 appeared as a yellow oil in the distillate. The oil was twice extracted with benzene (20 ml.). No attempt was made to measure the amount of radioactive product, but in base runs the yields on this step averaged 63%.

(5) 3-Chloroacetanilide-Cl-36.--3-Chloroaniline-Cl-36 in 20 ml. benzene was heated to 60° and 1 ml. acetic anhydride added. The benzene was evaporated down to one-half volume, 4 ml. water was added, and the remainder of the benzene was removed by distillation. The crystalline product, m. p., 69-71°, (lit. 72-73°) (8) was recrystallized from 25% ethanol until the activity of the recrystallizing solvent was constant (110) and the melting point was raised to 72°. Yield; 1.1 g., 48.5% based on 3-nitrobenzenemercuric acetate. Specific activity of the purified compound was 77,000 d/m/mg.; 38 microcuries, or 19% (38% of theory) of the original activity, was utilized.

2. Preparation of Metabolites

a) Possible Metabolites of 4-Chloroacetanilide

(1) 4-Chloroacetanilide.--4-Chloroacetanilide was purchased from Eastman Chemical Company and was recrystallized

from 50% ethanol to a sharp melting point of 178° (44).

(2) 4-Chloroaniline.--4-Chloroaniline was purchased from the Eastman Chemical Company and was recrystallized from 50% ethanol to a sharp melting point of 72° (7).

(3) 2-Hydroxy derivatives

(a) 4-Chloro-2-hydroxyaniline.--The procedure of Hodgson and Moore (49) was employed to make the intermediate 4-chloro-2-hydroxynitrobenzene for the preparation of 4-chloro-2-hydroxyaniline. Forty grams of concentrated H_2SO_4, 29 g. of sodium nitrate, and 70 ml. of water were mixed together and left to stand 4 hours in a 500 ml. 3-necked flask fitted with thermometer, stirrer, and dropping funnel. The reaction mixture was cooled to 18° and 24 g. of 3-chlorophenol (Eastman) was added dropwise over a period of one hour. Constant stirring was maintained during the period of addition. After addition of the 3-chlorophenol was completed, the mixture was stirred in the cold for two hours and was then diluted with two volumes of water. The supernatant liquid was decanted from the solid portion, the solid was water washed, and was steam distilled. A yellow oil solidified in the distillate. This solid was pressed on a Buchner funnel and was again steam distilled. Pure 4-chloro-2-hydroxynitrobenzene, m.p. 41°, was obtained. The yield was not high. The average of several runs was 3.8 g., 13%. However, a second product, 2-chloro-4-hydroxybenzene is also formed in this reaction. This remained in the reaction flask during steam distillation of the 2-hydroxy compound and was employed in the preparation of 2-chloro-4-hydroxyaniline [III, A, 2, c), (3), (a)].

Three and eight-tenths grams of 4-chloro-2-hydroxynitrobenzene was dissolved in hot 10% NaOH. The solution turned a vivid scarlet color. Solid sodium hyposulfite ($Na_2S_2O_4$) was gradually added to the hot solution until all the color was discharged. The solution was cooled, neutralized with H_2SO_4, and extracted with ether. After evaporation of the solvent, the gray-black crystalline 2-hydroxy-4-chloroaniline was recrystallized several times from water containing a little sodium bisulfite. White crystals, m. p. 153-154° (lit. 154°) resulted (48). Average yield of purified compound; 1.1 g., 35%.

(b) 4-Chloro-2-hydroxyacetanilide.--4-Chloro-2-hydroxyaniline was dissolved in benzene and heated to 50°. Molar quantities plus 15% excess acetic anhydride was added and the solvent was evaporated down to 1/2 volume. Water was added and the remainder of the benzene evaporated off. The product, white and crystalline, precipitated out upon cooling. The compound was recrystallized from a minimum of 70% ethanol. Average yields of the purified compound were about 80%, m. p. 186° (lit. 186°) (48).

b) Possible Metabolites of 3-Chloroacetanilide

(1) 3-Chloroacetanilide.--Eastman Chemical Company's commercial 3-chloroacetanilide was purified by recrystallization from 50% ethanol to a sharp melting point of 73° (8).

(2) 3-Chloroaniline.--Eastman's technical grade 3-chloroaniline was purified by distillation. The fraction distilling at 226-7° at atmospheric pressure was taken (lit. 229.8) (7). The compound was clear and colorless. It was stored in the dark until used to guard against discoloration from oxidation.

(3) 4-Hydroxy derivatives.

(a) 3-Chloro-4-hydroxyaniline.--Technical grade (Eastman) 3-chloro-4-hydroxynitrobenzene was dissolved in 10% NaOH. The solution, a red-orange color, was heated to reflux temperature. Solid sodium hyposulfite was added until all color was gone. The colorless solution was then cooled, neutralized with H_2SO_4 and left in the cold room. The reduced compound precipitated, and was filtered. Recrystallization from water and sodium bisulfite yielded a white compound, m. p. 152-153° (lit. 154°) (29). Yields averaged 77%.

(b) 3-Chloro-4-hydroxyacetanilide.--3-Chloro-4-hydroxyaniline, was dissolved in benzene, acetylated with molar quantities of acetic anhydride plus 15% excess. The solvent had to be entirely removed before the compound would crystallyze. Recrystallization from 25% ethanol yielded a sharply melting compound, m. p. 143° (lit. 144°) (29). Average yields were 70% of the purified compound.

(4) 6-Hydroxy derivatives.

(a) 3-Chloro-6-hydroxyaniline.--Technical grade (Eastman) 3-chloro-6-hydroxynitrobenzene was dissolved in 10% NaOH. The deep yellow solution was heated to reflux temperature and solid sodium hyposulfite added until the color was discharged. The reduction was very rapid. The solution was neutralized and cooled. 3-Chloro-6-hydroxyaniline precipitated out immediately. Recrystallization from water and sodium bisulfite did not yield a white compound, and it was found that the material tended to oxidize quickly. A rapid recrystallization from water with sodium hyposulfite and charcoal, however, yielded the gleaming white amine that did not discolor during a period of six weeks. The literature reports (71, 97) that this compound, when it can be made, is not stable. It is apparent that some contaminate is responsible for the instability and when 3-chloro-6-hydroxyaniline is obtained pure it is stable for an appreciable length of time. Yield of purified product (20%), m. p. 139-40° (lit. 138°).

(b) 3-Chloro-6-hydroxyacetanilide.--The acetylated 6-hydroxy compound was obtained in good yield by acetylation with acetic anhydride in benzene. M. P. 185° (lit. 185°) (71); yield, 80%.

c) Possible Metabolites of 2-Chloroacetanilide.

(1) 2-Chloroacetanilide.--Eastman Chemical Company's 2-chloroacetanilide was recrystallized from 50% ethanol to a sharp melting point of 88° (8).

(2) 2-Chloroaniline.--Eastman's 2-chloroaniline was purified by distillation. The fraction boiling at 206-7° at atmospheric pressure was taken. The liquid was clear and colorless. Lit. b.p., 208.8° (8).

(3) 4-Hydroxy derivatives.

(a) 2-Chloro-4-hydroxyaniline.--The intermediate in the preparation of 2-chloro-4-hydroxyaniline was prepared in conjunction with 4-chloro-2-hydroxynitrobenzene (49) [see III, A, 2, a), (3), (a)] . 2-Chloro-4-hydroxynitrobenzene remained in the flask as a brown, oily solid, m. p. 80-106° after 4-chloro-2-hydroxynitrobenzene had been distilled. Recrystallization from water and dilute HCl yielded white, feathery needles, m. p. 121-122° (lit. 122°) (49). Yield of pure compound from 24 g. 3-chlorophenol; 9 g. (26%).

2-Chloro-4-hydroxynitrobenzene (8.8 g.) was dissolved in 10% NaOH. The orange-brown solution was brought to reflux temperature. Solid sodium hyposulfite was gradually added until all the color disappeared. The colorless solution was cooled, neutralized with H_2SO_4, and extracted with ether. Recrystallization of the compound from water to which sodium bisulfite had been added yielded 3.2 g. (46.7%) of white, platelike crystals, m. p. 160° (lit. 160°) (48).

(b) 2-Chloro-4-hydroxyacetanilide.--2-Chloro-4-hydroxyaniline was dissolved in benzene and acetylated at 60° with equal molar quantities of acetic anhydride plus 15% excess. The resulting compound was recrystallized from absolute ethanol with a little charcoal present. The melting point, 124-5°, was sharp but higher than that reported in the literature (53), 121° (53). Infra red analysis showed one strong carbonyl band at 5.9 microns. This indicated that only one acetyl group was present. A broad medium band centered at 3 microns indicated the presence of a free hydroxy group. The compound was soluble in dilute NaOH also indicating that the hydroxyl group was unsubstituted. Calc. for Cl, 19.1%; for N, 7.5%. Found for Cl, 19.7%; N, 7.5%. These data were taken as sufficient evidence that the compound melting at 124-5° was 2-chloro-4-hydroxyacetanilide.

(4) 6-Hydroxy derivatives.

(a) 2-Chloro-6-hydroxyaniline.--The intermediate, 2-chloro-6-hydroxynitrobenzene, for the preparation of 2-chloro-6-hydroxyaniline, was made by the method of Hodgson and Moore (49). 3-Chlorophenol (12.8 g.) was slowly added to 75 g. oleum (27%, SO_3) and cooled after each addition. The mixture was kept for 12 hours and then heated for two hours on a steam bath to complete sulfonation in the 4- and 6-positions. Nitric acid (4.5 ml.; d. 1.5) was dissolved in oleum and was added during a period of 15 minutes to the cooled mixture which was then left to stand two hours at room temperature. The solution was diluted about 1:1 with water and distilled in a current of super-heated steam. Extreme care had to be taken at this point, because distillation was not effected at a temperature below 155°. Charring took place, however, if the temperature went above 160°. As hydrolysis of the disulfonated compound was effected, a viscous, yellow oil came over and solidified in the receiving flask, m. p. 34°. Recrystallization of the material from hexane yielded a colorless compound, m. p. 36° (lit. 37.5°). Yields averaged about 7 g. (50%).

2-Chloro-6-hydroxynitrobenzene was dissolved in 10% NaOH and brought to reflux temperature. Solid sodium hyposulfite was added gradually until all the deep red color disappeared. The solution was cooled and made neutral with H_2SO_4. The amine precipitated out of the neutral solution, was filtered, and was recrystallized from water with sodium bisulfite and charcoal present. The final product was a lustrous, white crystalline material melting at 122° (lit. 122°) (48).

(b) 2-Chloro-6-hydroxyacetanilide.--Pure 2-chloro-6-hydroxyaniline, m. p. 122°, was put into water and conc. HCl added dropwise until the compound dissolved. The solution was warmed to 50° and molar quantities plus 15% excess acetic anhydride was added and the solution stirred vigorously. Immediately sufficient saturated sodium acetate solution was added to bring the solution to neutral and precipitate the product. After cooling, the product was filtered, thoroughly washed with water, and recrystallized from 50% ethanol, m. p. 190-191°. Calc. for Cl, 19.13%. Found, 19.25%. The possibility was considered that acetylaction may have been on the hydroxy group or that ring closure may have occurred. Either situation would give a chlorine analysis close to that for the N-acetyl compound. However, infra red analysis revealed one strong carbonyl band at 6 microns and a broad, medium band centered at 3.2 microns. The band at 3.2 microns indicated that the hydroxy group was not substituted. Hence, acetylation was on the amino group and there was no ring closure.

B. Metabolism Studies

In each experiment, male Sprague-Dawley rats were injected with one of the radioactive chloroacetanilides, and the urine was collected for 72 hours. The activity of the urine was checked each 4 hours by plating 0.1 ml. of urine on duplicate planchets. The most active urine samples were pooled to be used in isotope dilution experiments. The general method was to add a carefully weighed amount (approximately 0.2 g.) of the postulated metabolite to an aliquot of radioactive urine and to bring the inactive compound into equilibrium with any chemically identical radioactive material in the urine sample. The metabolite was then purified by recrystallization.

To determine the specific activity of the recrystallized samples, about 5 mg. (carefully weighed) was diluted to 1 ml. in 95% ethanol. Exactly 0.2 ml., or approximately 1 mg., was plated on duplicate planchets in an almost infinitely thin layer. These planchets were plated and counted at various stages of recrystallization and recorded in counts per minute per milligram (c/m/mg.). The recrystallizations were continued until the specific activity was constant. In some cases where several recrystallizations were made from the same solvent, activity of the recrystallizing medium was determined. The metabolite was dissolved, allowed to crystallize slowly in a centrifuge tube, set for 12 hours at 5°, and then centrifuged for 5 minutes. The supernatant was removed immediately with a filter stick and 0.1 ml. plated on duplicate planchets. Care was taken so that each sample was plated at the same temperature insuring equal saturation of the solvent with the solute. When the activity of the recrystallizing medium was constant, the compound was assumed to be pure and the specific activity was determined.

All planchets were counted for at least 4840 counts or, if activity was low, for a total of at least two hours.

Background was counted three or more times within a 24 hour period. All measurements were made in a Nuclear Corporation of Chicago, Model D-47, thin window, Q-gas counting chamber with a Model C-110 automatic sample changer, and Model 183 scaler with an efficiency of 20% as calculated against a Co-60 standard.

1. Identification of Metabolites of 4-Chloroacetanilide.

Two Sprague-Dawley male rats each weighing 470 g. were injected with 100 mg./kg. body weight of 4-chloroacetanilide-Cl-36 (40) (34,000 c/m/mg.) in 2% Methocel suspension. Each rat was placed in a metabolism cage. Urine was collected in a vial which was packed in dry ice, so that the urine was frozen as soon as it was delivered to the vial. The vials were changed every four hours allowing the urine no opportunity to thaw. Thus, bacterial or enzymic action was prevented. The urine from the first 40 hours was taken and the specific activity recorded as 25,500 c/m/ml. A 4 ml. aliquot was set aside to measure the presence of chloride ion in the urine. Eight 5 ml. aliquots were measured out to determine the presence of unchanged 4-chloroacetanilide and these seven postulated metabolites of 4-chloroacetanilide-Cl-36:

4-chloroaniline,
4-chloro-2-hydroxyacetanilide,
glucuronide of 4-chloro-2-hydroxyacetanilide,
ethereal sulfate of 4-chloro-2-hydroxy-acetanilide,

4-chloro-2-hydroxyaniline,
glucuronide of 4-chloro-2-hydroxyaniline,
ethereal sulfate of 4-chloro-2-hydroxyaniline.

a) 4-Chloroacetanilide

Non-radioactive 4-chloroacetanilide (m. p. 178°; 225.1 mg.) was placed in a 25 ml. Erlenmeyer flask and dissolved in 95% ethanol. A 5 ml. aliquot of radioactive urine (25,500 c/m/ml.) was added to the solution of 4-chloroacetanilide. A stirring bar was placed in the flask and the mixture was agitated with a magnetic stirrer for one hour at room temperature. Yellowish, 4-chloroacetanilide weighing 204.4 mg. (m. p. 169-170°) was filtered out of the mixture.

The material was recrystallized from 50% ethanol and dried over P_2O_5. The micro balance was employed to weigh out 9.38 mg. of 4-chloroacetanilide. This was dissolved in 2 ml. 95% ethanol and duplicate planchets were plated with 0.2 ml. or 0.938 mg. 4-chloroacetanilide. Activity was 18 and 17 c/m/mg.

Changing the chemical species is regarded as an excellent method for purification of radioactive materials. For this reason, the compound was deacetylated by refluxing in 15 ml. of 20% NaOH for one-half hour. Previous investigation had shown that this treatment was sufficient to hydrolyze the acetyl group. The solution was cooled, neutralized with H_2SO_4 and extracted three times with benzene.

The benzene solution was heated to 50°, one ml. acetic anhydride was added, and the benzene distilled off to one-half volume. Six ml. water was added and the remainder of the benzene distilled off. Upon cooling white crystals precipitated. These crystals were dried over P_2O_5. M. p. 176-177° (44).

One ml. 95% ethanol was used to dissolve 5.42 mg. of the crystalline 4-chloroacetanilide, and 0.2 ml. or 1.08 mg. was plated on duplicate planchets. Recorded counts were 13.4 and 13.7 c/m/mg.

Recrystallization in 50% methanol, 50% acetic acid, and 50% ethanol yielded products with constant activity:

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
50% ethanol	0.94	18.0	17.0
Benzene	1.08	13.4	13.7
50% methanol	1.08	12.8	12.9
30% acetic acid	1.05	13.4	12.6
50% ethanol	0.98	14.4	11.8

The average of the last four determinations is 13.1 c/m/mg. Therefore,

$$\frac{13.1 \text{ c/m/mg.} \times 225.1 \text{ mg.} \times 100}{127{,}500 \text{ c/m}} = 2.31\%,$$

unchanged 4-chloroacetanilide in the urine.

To test if all active 4-chloroacetanilide has been extracted from the radioactive urine, the process was repeated. The same aliquot was added to 196.0 mg. 4-chloroacetanilide dissolved in 95% ethanol. The mixture was refluxed five hours while stirred by a magnetic stirrer. After cooling, 159.1 mg. of 4-chloroacetanilide was brought down, m. p. 175°. The material was treated in the same manner as for the first determination and brought to constant activity:

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
50% ethanol	0.96	13.8	14.4
Benzene	0.89	8.1	9.4
50% methanol	0.91	8.0	8.7
30% acetic acid	0.96	7.8	7.9

The average of the last three determinations is 8.3 c/m/mg.

In the original mixture, 225.1 mg. had been added and 204.4 mg. was recovered. The material that was left in solution was, therefore, 20.7 mg. At 13.1 c/m/mg., this equals 271.2 counts. In the second treatment, 196.0 mg. had been added. At 8.3 c/m/mg. this is 1626.7 counts. The amount not accounted for by the first treatment was: 1627 - 271 or 1356 counts. Therefore, equilibrium was not achieved between the 4-chloroacetanilide added and the 4-chloroacetanilide-Cl-36 in solution by stirring one hour at room temperature. It became evident that more drastic methods were necessary. Apparently, the method used (102) is not adequate for equilibration of this compound.

A third treatment was given the urine to make certain that refluxing for five hours was sufficient to isolate all active 4-chloroacetanilide. 4-Chloroacetanilide (194.7 mg.) was dissolved in 95% ethanol. Treatment was identical to that for the second determination. The final activity of the compound was 1.4 and 1.3 c/m/mg. as measured on duplicate planchets.

Whether this amount of activity was to be expected from the material left in solution was determined as follows:

In the first treatment, 225.1 mg. - 204.4 mg. or 20.7 mg. was left in solution. In the second treatment, 196.0 mg. - 159.1 mg. or 36.9 mg. was left in solution. This is a total of 57.6 mg. with a specific activity of 8.3 c/m/mg. or 478 counts for the entire aliquot. In the third determination, 194.7 mg. + 57.6 mg. from first and second determination or 252.3 mg., was the total amount of 4-chloroacetanilide in solution. This equals 478 counts per 252.3 mg. or 1.9 c/m/mg. Since the measured activity was only 1.4 and 1.3 c/m/mg., it can be assumed that equilibration of all active 4-chloroacetanilide was completed by refluxing for five hours with base compound. The percentage of activity recovered from the first two determinations was 2.31% and 1.90% or a total of 4.21%.

In order to determine quantitatively the amount of unchanged 4-chloroacetanilide, a new 5 ml. aliquot of urine with a specific activity of 46,700 c/m was added to 205.8 mg. of 4-chloroacetanilide dissolved in acetone. This solution was stirred with a magnetic stirrer at reflux temperature for five hours. After the mixture was set in the freezer overnight, 124.9 mg. 4-chloroacetanilide precipitated. This material was refluxed thirty minutes in 10% NaOH. It was cooled, made neutral with H_2SO_4, extracted in 15 ml. benzene, and reacetylated in the extraction medium. After the benzene was distilled off, yellowish crystals of 4-chloroacetanilide, m. p. 175-6° remained.

Exactly 4.84 mg. of this material was weighed out and diluted to one ml. with 95% ethanol. Duplicate planchets were plated with 0.2 ml. or 0.96 mg. of material. The resulting counts of 15.1 and 15.6 c/m/mg. were recorded.

The compound was next recrystallized in 50% methanol with charcoal present. The product came out white and crystalline, m. p. 178°. One mg. was plated on duplicate planchets and a specific activity of 9.9 and 11.3 or an average of 10.6 c/m/mg. was recorded.

Two successive recrystallizations with 50% ethanol and subsequent plating of the supernatant revealed that the compound was to radioactive purity (110). The compound was then plated on duplicate planchets with an average activity of 10.5 c/m/mg. recorded.

There were 205.8 mg. of base material added and 46,700 c/m in the urine aliquot. Therefore, the percent of 4-chloroacetanilide remaining unchanged in the urine was:

$$\frac{205.8 \text{ mg. x } 10.5 \text{ c/m/mg. x } 100}{46{,}700 \text{ c/m}} = 4.63\%,$$

unchanged 4-chloroacetanilide.

b) 4-Chloroaniline

A 5 ml. aliquot of urine (46,700 c/m/aliquot) was added to 197.6 mg. of 4-chloroaniline (m. p. 72°) dissolved in acetone. The material was refluxed for five hours and placed in the freezer overnight. Crude material weighing 90.8 mg. (m. p. 68-69°) precipitated and was recrystallized in 50% ethanol. The recrystallized material was acetylated in benzene with acetic anhydride. The product was yellowish in color (m. p. 174-6°). The compound was recrystallized in 30% methanol with charcoal and came out white and pure and melted sharply at 178° (44). Exactly 0.90 mg. of the purified compound was plated on duplicate planchets and gave a specific activity of 5.3 c/m/mg. Three recrystallizations from ethanol yielded a product that was radioactively pure (110) and had a specific activity of 4.5 c/m/mg.

Conversion of 197.6 mg. of base 4-chloroaniline to its acetylated equivalent yields 262.7 mg. This was the form in which the specific activity was read. Therefore,

$$\frac{262.7 \text{ mg. x } 4.5 \text{ c/m/mg. x } 100}{46{,}700 \text{ c/m}} = 2.53\%,$$

amount of 4-chloroaniline present in urine of rats injected with 4-chloroacetanilide-Cl-36.

c) 2-Hydroxy Derivatives

Since it was felt that enzymatic hydrolysis of ethereal sulfates and glucuronides may not be sufficient to assure complete conversion to the unconjugated phenols, a determination for total 2-hydroxy compounds was made to obtain a quantitative estimation.

Five ml. of radioactive urine (39,300 c/m/aliquot) was put into 5 ml. 10 N H_2SO_4 and the mixture refluxed one hour and 20 minutes to assure removal of the acetyl, sulfate, and glucuronide moieties. The solution was then cooled and made neutral with NaOH. 4-Chloro-2-hydroxyaniline (204.6 mg., m. p. 153°) dissolved in acetone was added and the mixture refluxed for five hours. At the end of the refluxing period, the reaction mixture was cooled, Na_2SO_4 was removed by filtration and twice washed with acetone to remove any 4-chloro-2-hydroxyaniline. The acetone was distilled from the filtrate and the remaining liquid frozen. Brownish crystals weighing 101.3 mg. precipitated.

Duplicate planchets were plated and a specific activity of 119.6 and 120.4 c/m/mg. was recorded.

The material was recrystallized in water with sodium hyposulfite present, but the compound was still brown. The liquid was removed by a filter stick; water, sodium hyposulfite, and charcoal were employed to recrystallize the product. When the charcoal was filtered off the compound crystallized out of solution in white, shiny needles (m. p. 152°, lit. 154°) (48).

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
Water + $Na_2S_2O_4$	0.94	119.6	120.4
Water + $Na_2S_2O_4$ + C	0.94	104.1	98.9

Three successive recrystallizations from water brought the activity of the plated filtrates to a constant count.

Recrystallization (from water)	c/m/planchet (duplicates)	
5	13.1	15.3
6	14.0	16.1

Material from the sixth recrystallization was dried over H_2SO_4 (m. p. 154°). Duplicate planchets were plated with 0.96 mg. with recorded average count of 119.1 c/m/mg.

$$\frac{294.6 \text{ mg. x } 119.1 \text{ c/m/mg. x } 100}{39{,}300 \text{ c/m}} = 61.96\%,$$

excreted as 2-hydroxy metabolites of 4-chloroacetanilide.

(1) 4-Chloro-2-hydroxyacetanilide

(a) Unconjugated 4-chloro-2-hydroxyacetanilide.--The presence of unconjugated 4-chloro-2-hydroxyacetanilide was detected by weighing out 0.2016 g. of the base compound and dissolving it in 95% ethanol in a 25 ml. Erlenmeyer flask.

A 5 ml. aliquot of radioactive urine (127,500 c/m) was added and stirred one hour and a half at room temperature. The compound was kept in solution by addition of sufficient ethanol. 4-Chloro-2-hydroxyacetanilide was precipitated by cooling the solution to 0°. Recrystallizations in 30% methanol, 25% acetic acid, and four recrystallizations from 50% ethanol yielded a product of constant specific activity of 15.5 c/m/mg.

Recrystallization medium	Mg. plated per planchet	c/m/mg. (duplicates)	
30% methanol	0.40	20.0	19.0
25% acetic acid	0.86	16.8	16.8
50% ethanol	0.94	18.3	17.7
50% ethanol	0.92	17.0	16.0
50% ethanol	0.92	15.5	15.4
50% ethanol	0.91	16.0	15.1

Average specific activity from the last two recrystallizations was 15.5 c/m/mg.

$$\frac{15.5 \text{ c/m/mg.} \times 201.6 \text{ mg.} \times 100}{127{,}500 \text{ c/m}} = 2.45\%,$$

unconjugated 4-chloro-2-hydroxyacetanilide.

(b) Glucuronide of 4-chloro-2-hydroxyacetanilide.--beta-Glucuronidase (Sigma Company, St. Louis; Bacterial Powder, 25,000 units/gram) was employed to hydrolyze the glucuronides. One unit of this bacterial powder frees one gamma of glucuronide from its conjugate (82). The amount of beta-glucuronidase necessary for the amount of radioactive urine used was determined as follows: The urine had a specific activity of 25,500 c/m/ml. and the injected compound contained 34,000 c/m/mg. There was, therefore, about 25,500 c/m/ml. / 34,000 c/m/mg. or 0.75 mg. of radioactive compound per ml. of urine. Assuming that at most one-third of the injected 4-chloroacetanilide-Cl-36 is in the form of the glucuronide of 4-chloro-2-hydroxyacetanilide, there would be 0.25 mg./ml. of 4-chloro-2-hydroxyacetanilide conjugated as the glucuronide. If there are 250 gamma (0.25 mg.) of the glucuronide per ml., then, there will be 5 x 250 gamma or 1250 gamma per aliquot. beta-Glucuronidase will hydrolyze 25,000 gamma per gram of enzyme. Then, 1250 gamma/aliquot / 25,000 gamma / gram of enzyme = 0.050 gram of enzyme per aliquot of urine needed to hydrolyze the glucuronide. Sigma recommends a one percent solution of the bacterial powder, hence, the enzyme solution was made up 0.1 g. / 10 ml.

Five ml. pH 6.8 phosphate buffer, 5 ml. 1% solution beta-glucuronidase, and 5 ml. radioactive urine from rats injected with 4-chloroacetanilide-Cl-36 was incubated together for 24 hours at 37.5^{0}. This mixture was filtered to remove any undissolved enzyme and added to 181.4 mg. 4-chloro-2-hydroxyacetanilide dissolved in 95% ethanol. The mixture was stirred for one hour at room temperature and set for 12 hours at 5°. The product that precipitated was recrystallized from 30% methanol, 25% acetic acid, and 50% ethanol.

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
30% methanol	0.83	50.0	50.7
25% acetic acid	0.99	52.4	53.4
50% ethanol	0.95	48.5	46.5
50% ethanol	0.91	47.5	45.3

A compound with a specific activity (average) of 47.5 c/m/mg. was obtained.

$$\frac{47.5 \text{ c/m/mg.} \times 181.4 \text{ mg.} \times 100}{127{,}500 \text{ c/m}} = 6.80\%,$$

unconjugated plus the glucuronide of 4-chloro-2-hydroxyacetanilide. The determination of unconjugated 4-chloro-2-hydroxyacetanilide yielded 2.45%. Therefore, 6.80% - 2.45% = 4.35%, excreted as the glucuronide of 4-chloro-2-hydroxyacetanilide.

(c) Ethereal sulfate of 4-chloro-2-hydroxyacetanilide.--Takadiastase (Parke, Davis and Company), a plant sulfatase, was used to hydrolyze the ethereal sulfates from their conjugates in a manner similar to that used for the hydrolysis of the glucuronides (102).

Five ml. of a one percent solution of taka-diastase, 5 ml. of pH 6.8 phosphate buffer, and 5 ml. of urine from rats injected with 4-chloroacetanilide were incubated for

24 hours at 37.5°. The hydrolyzed urine was added to 155.0 mg. 4-chloro-2-hydroxylacetanilide (m. p. 190°), dissolved in ethanol and stirred at room temperature for one hour. Sufficient ethanol was added to keep the compound in solution during the stirring. The mixture was placed in the cold room to induce precipitation of the compound.

Recrystallization of the material in 50% methanol yielded a compound of m. p. 189-190° with a specific activity of 33 c/m/mg. Subsequent recrystallizations in 30% acetic acid and 50% ethanol yielded a compound with a specific activity of 25.5 c/m/mg.

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
50% methanol	0.99	34.2	31.6
25% acetic acid	0.89	29.5	29.5
50% ethanol	0.97	30.0	30.6
50% ethanol	0.96	25.5	25.6
50% ethanol	0.97	24.8	26.1

$$\frac{25.5 \text{ c/m/mg.} \times 155.0 \text{ mg.} \times 100}{127{,}500 \text{ c/m}} = 3.1\%,$$

unconjugated plus the ethereal sulfate of 4-chloro-2-hydroxyacetanilide. The determination for the unconjugated metabolite yielded 2.45%. Therefore, 3.10% - 2.45% = 0.65%, identified as the ethereal sulfate of 4-chloro-2-hydroxyacetanilide.

(2) 4-Chloro-2-hydroxyaniline

(a) Unconjugated 4-chloro-2-hydroxyaniline.-- The presence of the unconjugated but deacetylated and hydroxylated metabolite, 4-chloro-2-hydroxyaniline, was determined as follows: 208.1 mg. of base 4-chloro-2-hydroxyaniline (m. p. 153°) was dissolved in ethanol and a 5 ml. aliquot of radioactive urine (127,500 c/m/aliquot) was added. The solution was stirred for one and a half hours and set in the cold room. The 152.7 mg. of material that precipitated was recrystallized in water with sodium hyposulfite present to prevent oxidation of the amine. Subsequent recrystallizations from 50% ethanol and 30% methanol were carried out.

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
Water	1.08	18.8	----
50% ethanol	0.97	9.8	9.6
30% methanol	0.92	7.1	8.5

Two further treatments with water and sodium hyposulfite yielded a compound of constant activity when 0.2 ml. of the recrystallizing fluid from the water treatments was plated.

Recrystallizing medium	Volume plated	c/m/planchet (duplicates)	
Water	0.2 ml.	3.4	3.1
Water	0.2 ml.	3.2	3.2

A final plating of the compound yielded a specific activity of 4 c/m/mg.

Percentage of 4-chloro-2-hydroxyaniline detected:

$$\frac{4 \text{ c/m/mg.} \times 207.1 \text{ mg.} \times 100}{127,500 \text{ c/m}} = 0.65\%$$

unconjugated.

(b) Glucuronide of 4-chloro-2-hydroxyaniline.--The determination of the presence of the glucuronide of the deacetylated compound was similar to that previously discussed for the glucuronide of 4-chloro-2-hydroxyacetanilide, [III, B, 1, c), (1), (b)]. Five ml. of pH 6.8 phosphate buffer, 5 ml. of 1% beta-glucuronidase, and 5 ml. of urine from rats injected with 4-chloroacetanilide-Cl-36 were incubated together for 24 hours at 37.5°. The incubated mixture was then stirred with 212.4 mg. of 4-chloro-2-hydroxyaniline for one hour at room temperature. Alcohol was added to keep the material in solution. The compound precipitated out of solution after 10 hours at 5°. Recrystallizations were made from hot water plus sodium hyposulfite, from 50% ethanol, and twice again from water.

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
Water	0.89	14.7	11.8
50% ethanol	1.03	6.1	8.8
Water	0.96	5.1	5.5
Water	1.01	4.3	6.5

Average of the last two recrystallizations was 5.3 c/m/mg.

$$\frac{212.4 \text{ mg.} \times 5.3 \text{ c/m/mg.} \times 100}{127{,}500 \text{ c/m}} = 0.88\%$$

Unconjugated 4-chloro-2-hydroxyaniline was found in an amount equal to 0.65%; 0.88% - 0.65% = 0.23% excreted as the glucuronide of 4-chloro-2-hydroxyaniline.

(c) Ethereal sulfate of 4-chloro-2-hydroxyaniline.--Five ml. 1% takadiastase solution, 5 ml. pH 6.8 phosphate buffer, and 5 ml. radioactive urine (127,500 c/m/aliquot) were incubated together for 24 hours at 37.5°. This mixture was added to 223.1 mg. of 4-chloro-2-hydroxyaniline and stirred at room temperature for one and one-half hours. The mixture was allowed to stand at 5° and the compound precipitated.

The material was recrystallized in 50% ethanol and then in water with sodium bisulfite present, and the compound was plated on duplicate planchets.

Recrystallizing medium	Mg. plated per planchet	c/m/mg. (duplicates)	
50% ethanol	0.95	26.1	30.2
water	1.03	15.6	17.5

The filtrates from subsequent water with sodium bisulfite recrystallizations were plated until constant activity of the recrystallizing medium was reached. The compound from the last recrystallization was plated and 13.6 c/m/mg. were recorded.

$$\frac{13.6\ \text{c/m/mg.} \times 223.1\ \text{mg.} \times 100}{127{,}500\ \text{c/m}} = 2.38\%,$$

4-chloro-2-hydroxyaniline, unconjugated and as the ethereal sulfate. Unconjugated 4-chloro-2-hydroxyaniline was found in an amount equal to 0.65%. Therefore, 2.38% - 0.65% = 1.73%, ethereal sulfate of 4-chloro-2-hydroxyaniline excreted in the urine.

d) Inorganic Chloride Ion

The presence and amount of inorganic chloride ion in the urine of rats injected with 4-chloroacetanilide was determined as follows: A four ml. aliquot (25,500 c/m/ml.) was put into a 15 ml. conical centrifuge tube and acidified with dilute HNO_3. The urine was heated in a water bath and $AgNO_3$ solution (0.1 N) was added dropwise. Coagulation of the formed AgCl was induced by stirring and heating. When no further precipitation occurred, the solid in the tube was spun down in the centrifuge. The supernatant was tested for unprecipitated chloride ion and was discarded when none was detected. The precipitated AgCl was washed three times with 6 ml. water, once with ethanol, and then dissolved in 12 N NH_4OH and filtered by means of a filter stick into a clean tube. The filter stick was thoroughly washed with ammonia water. The AgCl was reprecipitated with HNO_3, and the washing, dissolution, and transferring to a clean tube was repeated twice, the last time into a tared tube. The finally precipitated AgCl was dried at 120° for four hours.

The weight of AgCl was 28.3 mg. The AgCl was dissolved in 2.83 ml. 12 N NH_4OH and 0.1 ml. or 1 mg. was plated on each of two planchets, and the activity recorded.

The entire process was repeated twice on this sample of AgCl. The activity of the second and third determinations was very nearly identical.

Second specific activity: 42.2 and 42.6 c/m/mg.

Third specific activity: 43.0 and 42.2 c/m/mg.

There were 28.3 mg. AgCl precipitated by $AgNO_3$ from 4 ml. urine or 28.3 x 42.6 = 1205.6 c/m/4 ml. of urine, or 301.4 c/m/ml.

$$\frac{301.4 \text{ c/m/ml.} \times 100}{25{,}500 \text{ c/m/ml.}} = 1.18\%,$$

activity present as chloride ion.

2. Identification of Metabolites of 3-Chloroacetanilide.

Three Sprague-Dawley male rats weighing 390, 410, and 420 g. were injected intraperitoneally with 3-chloroacetanilide-Cl-36 (100 mg./kg. of body weight) in a 2% Methocel suspension. Urine was collected from each rat as described in III, B, 1. Fifty-one ml. of the most active urine (26,000 c/m/ml.) was combined to make seventeen 3 ml. aliquots to be used in determining the percentage of unchanged compound, total 4- and 6-hydroxy metabolites, inorganic chloride ion, and the following postulated metabolites of 3-chloroacetanilide:

3-chloroaniline,
3-chloro-4-hydroxyacetanilide,
glucuronide of 3-chloro-4-hydroxyacetanilide,
ethereal sulfate of 3-chloro-4-hydroxyacetanilide,
3-chloro-4-hydroxyaniline,
glucuronide of 3-chloro-4-hydroxyaniline,
ethereal sulfate of 3-chloro-4-hydroxyaniline,
3-chloro-6-hydroxyacetanilide,
glucuronide of 3-chloro-6-hydroxyacetanilide,
ethereal sulfate of 3-chloro-6-hydroxyacetanilide,
3-chloro-6-hydroxyaniline,
glucuronide of 3-chloro-6-hydroxyaniline,
ethereal sulfate of 3-chloro-6-hydroxyaniline.

a) 3-Chloroacetanilide

Three ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was refluxed five hours with 217.4 mg. of 3-chloroacetanilide (m. p. 73°) dissolved in 95% ethanol. Solid material (m. p. 68-70°) precipitated when the solution was left 12 hours at 5°. The material was recrystallized from 50% ethanol, 50% methanol, 30% acetic acid, and four times from 25% ethanol. Duplicate planchets, plated with 0.1 ml. filtrate from each of the last four recrystallizations, indicated constant activity:

Recrystallization from 25% ethanol	c/m/planchet (duplicates)	
4	13.5	12.6
5	11.2	11.9
6	13.7	15.7
7	11.4	12.3

Specific activity planchets were plated with 1.08 mg. of 3-chloroacetanilide (m. p. 71-72°) from the last recrystallization to give 4.5 and 5.2 c/m/mg. or an average count of 4.8 c/m/mg.

$$\frac{4.8 \text{ c/m/mg.} \times 217.4 \text{ mg.} \times 100}{78{,}000 \text{ c/m}} = 1.34\%,$$

excreted as unchanged 3-chloroacetanilide.

b) 3-Chloroaniline

3-Chloroaniline (204.9 mg., b. p. 224°) was dissolved in 95% ethanol, added to 3 ml. urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36, and refluxed for 5 hours. The solution was cooled to 50° and HCl was added to form the amine hydrochloride. Acetic anhydride (1/2 ml.) and 3 ml. saturated sodium acetate were added in immediate succession. The precipitate melted at 64-68°. The crude 3-chloroacetanilide was recrystallized from 50% ethanol, 25% methanol, 25% acetic acid, and six times from 25% ethanol before constant activity of the recrystallizing medium was reached:

Recrystallization from 25% ethanol	c/m/planchet (duplicates)	
4	52.8	51.3
5	45.5	45.5
6	40.5	37.0
7	19.4	19.6
8	17.0	23.8
9	17.0	18.0

Two planchets were plated with 1.08 mg. dried material

(m. p. 71-72°) to give 27 and 28 c/m/mg. or an average of 27.5 c/m/mg.

The amount of 3-chloroaniline originally added to the radioactive urine was 204.9 mg. This is equivalent to 272.4 mg. of 3-chloroacetanilide, the form in which the compound was isolated.

$$\frac{272.4 \text{ mg.} \times 27.5 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 9.60\%,$$

isolated as 3-chloroacetanilide. Inasmuch as 1.34% was previously determined to have been excreted unchanged, 9.60% - 1.34% = 8.26%, excreted as 3-chloroaniline.

c) 4-Hydroxy Derivatives

Three ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was refluxed one hour with 3 ml. 10 N H_2SO_4 to hydrolyze the acetyl, glucuronide, and sulfate groups from the metabolites of 3-chloroacetanilide. The solution was cooled and neutralized with NaOH. The mixture was then refluxed 5 hours with 201.1 mg. 3-chloro-4-hydroxyaniline (m. p. 153°). When the mixture was cooled, the precipitate was washed with cold water. The Na_2SO_4 dissolved leaving dark brown crystals, which were recrystallized in water and sodium hyposulfite. The melting point of the product at this stage was 149°. The compound was recrystallized from 50% ethanol and then seven times from water containing sodium hyposulfite. The recorded activity from duplicate planchets plated with 0.1 ml.

recrystallizing medium did not reveal satisfactory constancy:

Recrystallization water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
5	142.3	138.4
6	110.3	129.4
7	158.9	123.8
8	128.7	117.8
9	154.3	149.7
10	146.0	140.0

Duplicate planchets were then plated with 0.83 mg. of 3-chloro-4-hydroxyaniline from the last recrystallization (m. p. 152°) to give an average count of 221 c/m/mg.

$$\frac{201.1 \text{ mg. x } 221 \text{ c/m/mg. x } 100}{78,000 \text{ c/m}} = 56.98\%$$

The product was then recrystallized once with 50% ethanol and then again with water and sodium hyposulfite. This recrystallization medium was plated (0.1 ml.) to give 157.5 and 158.7 c/m/planchet. Duplicate planchets were plated with 1.63 mg. of the dried compound from the last recrystallization to give 223.4 and 223.9 c/m/mg.

$$\frac{201.1 \text{ mg. x } 223.7 \text{ c/m/mg. x } 100}{78,000 \text{ c/m}} = 57.66\%,$$

total 4-hydroxy compounds. The difference between the two determinations of the specific activity of the total 4-hydroxy compounds was 0.68% or less than 2% error. The total

4-hydroxy metabolites was taken as the average of the two determinations: 57.32%, excreted as 4-hydroxy metabolites of 3-chloroacetanilide.

(1) 3-Chloro-4-hydroxyacetanilide

(a) Unconjugated 3-chloro-4-hydroxyacetanilide.--3-Chloro-4-hydroxyacetanilide (182.3 mg., m. p. 143°) was dissolved in 95% ethanol and refluxed 5 hours with 3 ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36. The precipitate that formed when the solution cooled was recrystallized from 25% ethanol, 25% acetic acid, 25% methanol and then six times from 25% ethanol to constant radioactivity of the recrystallizing medium:

Recrystallization from 25% ethanol		c/m/planchet (duplicates)	
4		41.2	38.6
5		30.0	28.2
6		43.2	59.1
7		28.4	27.8
8		29.9	31.3
9	(single planchet)	28.2	----

The product (m. p. 143-145°) from the last recrystallization was dried over H_2SO_4 and duplicate planchets were plated with 0.91 mg., each. The specific activity was 14.8 and 14.9 c/m/planchet or an average of 16.3 c/m/mg.

$$\frac{16.3 \text{ c/m/mg.} \times 182.3 \text{ mg.} \times 100}{78{,}000 \text{ c/m}} = 3.81\%,$$

excreted as unconjugated 3-chloro-4-hydroxyacetanilide.

(b) Glucuronide of 3-chloro-4-hydroxyacetanilide.--Three ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was incubated for 24 hours at 37.5° with 3 ml. 1% beta-glucuronidase (Sigma) and 3 ml. pH 6.8 phosphate buffer. This solution was refluxed 5 hours with 201.9 mg. 3-chloro-4-hydroxyacetanilide dissolved in 95% alcohol. The solid material recovered when the mixture was cooled to 5° and recrystallized from 25% ethanol, 25% methanol, 25% acetic acid, and 6 times from 25% ethanol:

Recrystallization from 25% ethanol		c/m/planchet (duplicates)	
4		169.0	182.0
5		79.6	86.8
6		47.4	48.3
7		127.0	136.4
8	(single planchet)	125.2	----
9		126.6	127.0

Specific activity planchets were plated with 0.81 mg. of product from the last recrystallization. Activity recorded was 58.8 and 61.0 c/m/planchet or 74 c/m/mg.

$$\frac{201.9 \text{ mg.} \times 74 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 19.16\%,$$

excreted as unconjugated plus the glucuronide of 3-chloro-4-hydroxyacetanilide. Previously 3.81% had been identified as the unconjugated metabolite; therefore, 19.16% - 3.81% = 15.35%, excreted as the glucuronide of 3-chloro-4-hydroxyacetanilide.

(c) Ethereal sulfate of 3-chloro-4-hydroxyacetanilide.--A three ml. aliquot of urine containing 78,000 c/m was incubated with 3 ml. 1 % takadiastase (Parke, Davis and Company) and 3 ml. pH 6.8 phosphate buffer for 24 hours at 37.5°. This mixture was refluxed five hours with 187.8 mg. 3-chloro-4-hydroxyacetanilide (m. p. 143°) dissolved in ethanol. The alcohol was then distilled off and solid material (m. p. 136-140°) precipitated at 5°. This solid was recrystallized from 25% ethanol, 25% acetic acid, 25% methanol, and then five times from 25% ethanol. Duplicate planchets were plated with 0.1 ml. from each of the last five recrystallizations:

Recrystallization from 25% ethanol		c/m/planchet (duplicates)	
4		157.4	142.5
5		59.2	56.6
6		66.6	59.6
7	(single planchet)	67.8	----
8		62.8	60.1

Specific activity planchets were plated with 0.90 mg. of dried material from the last recrystallization (m. p. 143°). The activity recorded was 114.0 and 112.3 c/m/planchet or an average 125.6 c/m/mg.

$$\frac{187.8 \text{ mg.} \times 125.6 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 30.24\%,$$

excreted as unconjugated and the ethereal sulfate of 3-chloro-4-hydroxyacetanilide. Unconjugated 3-chloro-4-hydroxyacetanilide has been determined to be 3.81%. Therefore, 30.24% - 3.81% = 26.43%, excreted as the sulfate of 3-chloro-4-hydroxyacetanilide.

(2) *3-Chloro-4-hydroxyaniline*

(a) Unconjugated 3-chloro-4-hydroxyaniline.--Three ml. urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was refluxed five hours with 201.6 mg. 3-chloro-4-hydroxyaniline (m. p. 153°). The precipitate was recrystallized from 25% ethanol, 25% methanol, 25% acetic acid, and five times from water with sodium hyposulfite present. Duplicate planchets were plated from the last five recrystallizations with 0.1 ml. of recrystallizing medium.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	6.0	7.4
5	3.5	4.0
6	6.3	4.0
7	6.5	6.5
8	6.0	5.6

Product from the last recrystallization was plated on two planchets (0.81 mg./planchet), specific activity counted, and recorded as 0.3 and 1.5 c/m/planchet or an average of 1.1 c/m/mg.

$$\frac{201.6 \text{ mg.} \times 1.1 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 0.28\%,$$

excreted an unconjugated 3-chloro-4-hydroxyaniline.

(b) Glucuronide of 3-chloro-4-hydroxyaniline.--Three ml. radioactive urine (78,000 c/m) that had been hydrolyzed with buffered 1% beta-glucuronidase solution was refluxed 5 hours with 210.4 mg. 3-chloro-4-hydroxyaniline. The solid that precipitated in the cold was recrystallized from 25% ethanol, 25% acetic acid, 25% methanol, and 5 times from water with sodium hyposulfite. As in previous determinations of the metabolites of 3-chloroacetanilide, 0.1 ml. from each of the last recrystallizations was plated on duplicate planchets and the activity counted.

Recrystallization from water + $Na_2S_2O_4$		c/m/planchet (duplicates)	
4		29.4	27.8
5		11.2	10.8
6		3.4	4.0
7	(single planchet)	2.7	----
8		3.1	3.8

Duplicate specific planchets were plated and activity determined to be 0.5 c/m/mg.

$$\frac{210.4 \text{ mg. } \times 0.5 \text{ c/m/mg. } \times 100}{78{,}000 \text{ c/m}} = 0.20\%$$

Unconjugated 3-chloro-4-hydroxyaniline had been determined to be 0.28%. No glucuronide of 3-chloro-4-hydroxyaniline was, therefore, detected.

(c) Ethereal sulfate of 3-chloro-4-hydroxyaniline.-- Three ml. urine (78,000 c/m) was incubated with three ml. buffered (pH 6.8) 1% takadiastase (Parke, Davis and Company) for 24 hours at 37.5°. This mixture was refluxed five hours with 201.1 mg. 3-chloro-4-hydroxyaniline (m. p. 153°). The precipitate was recrystallized in 50% ethanol, 25% acetic acid, 25% methanol and 5 times from water and sodium hyposulfite. The water recrystallizations were each plated (0.1 ml.) on duplicate planchets:

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	9.5	7.8
5	6.5	5.1
6	4.6	4.8
7	6.3	3.4
8	7.7	5.8

Duplicate planchets were plated with 0.86 mg. (m. p. 153°) of material from the last recrystallization. Recorded specific activity was 4.1 and 5.7 c/m/planchet or 4.8 and 6.6 c/m/mg., an average of 5.7 c/m/mg.

$$\frac{201.1 \text{ mg.} \times 5.7 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 1.47\%,$$

excreted as unconjugated and the ethereal sulfate of 3-chloro-4-hydroxyaniline. About 0.28% was previously determined to be unconjugated; therefore, 1.47% - 0.28% = 1.19%, ethereal sulfate of 3-chloro-4-hydroxyaniline.

d) 6-Hydroxy Derivatives

Three ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was refluxed one hour with 10 N H_2SO_4. The hydrolyzed urine was neutralized with NaOH. The mixture was refluxed five hours with 205.6 mg. of 3-chloro-6-hydroxyaniline (m. p. 139°). The precipitate that formed when the mixture cooled was washed with cold water to remove sodium sulfate and recrystallized from water, 50% ethanol, 50% methanol, and then five times from water. Two planchets were plated with 0.1 ml. from each of the last five recrystallizations:

Recrystallization from water	c/m/planchet (duplicates)	
4	47.2	43.4
5	41.3	41.8
6	34.1	28.3
7	33.8	35.5
8	34.2	32.8

Duplicate planchets were plated with 1.01 mg. of material from the last recrystallization (m. p. 139°). Specific activity was recorded as 102 and 101 c/m/planchet or an average of 100.5 c/m/mg.

$$\frac{205.6 \text{ mg.} \times 100.5 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 26.49\%,$$

excreted as 6-hydroxy derivatives.

(1) 3-Chloro-6-hydroxyacetanilide

(a) Unconjugated 3-chloro-6-hydroxyacetanilide.-- Three ml. radioactive urine (78,000 c/m) from rats injected with 3-chloroacetanilide-Cl-36 was refluxed 5 hours with 205.6 mg. of 3-chloro-6-hydroxyacetanilide (m. p. 185°) dissolved in 95% ethanol. At the end of the refluxing period, the alcohol was distilled off and the solution set for 12 hours at 5°. The precipitate was recrystallized from 50% ethanol, 50% methanol, 50% acetic acid, and five times from 50% ethanol to constant activity of the solvent:

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	15.9	13.2
5	7.8	8.6
6	5.2	5.0
7	5.6	6.5
8	5.4	5.1

Material from the last recrystallization (m. p. 185°), was plated on duplicate planchets (1.05 mg.). Activity counted was 7.8 and 6.9 c/m/planchet or 7.4 and 6.6 c/m/mg., an average of 7.0 c/m/mg.

$$\frac{7.0\ \text{c/m/mg.} \times 205.6\ \text{mg.} \times 100}{78{,}000\ \text{c/m}} = 1.85\%,$$

unconjugated 3-chloro-6-hydroxyacetanilide.

(b) Glucuronide of 3-chloro-6-hydroxyacetanilide.--Three ml. radioactive urine (78,000 c/m), 3 ml. 1% beta-glucuronidase (Sigma) solution, 3 ml. pH 6.8 buffer were incubated together for 24 hours at 37.5°. This mixture was refluxed 5 hours with 202.2 mg. 3-chloro-6-hydroxyacetanilide (m. p. 185°) dissolved in 95% ethanol. Excess alcohol was distilled off and solid material allowed to precipitate in the cold. The material was recrystallized from 50% ethanol, 50% methanol, 50% acetic acid, and five times from 50% ethanol to constant activity of the solvent:

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	14.9	15.6
5	6.4	6.1
6	3.7	4.4
7	6.3	3.0
8	6.3	5.8

Duplicate planchets, plated with 0.99 mg. of 3-chloro-6-hydroxyacetanilide from the last recrystallization had a specific activity of 16.5 and 17.0 c/m/planchet or 16.6 and 17.0 c/m/mg., an average of 16.8 c/m/mg.

$$\frac{16.8 \text{ c/m/mg.} \times 202.2 \text{ mg.} \times 100}{78{,}000 \text{ c/m}} = 4.36\%,$$

unconjugated plus the glucuronide of 3-chloro-6-hydroxyacetanilide. A previous determination for unconjugated 3-chloro-6-hydroxyacetanilide gave 1.85%. Therefore, 4.36% - 1.85% = 2.51%, excreted as the glucuronide of 3-chloro-6-hydroxyacetanilide.

(c) Ethereal sulfate of 3-chloro-6-hydroxyacetanilide.--Three ml. of urine from rats injected with 3-chloroacetanilide-Cl-36 was incubated for 24 hours at 37.5° with 3 ml. of 1% takadiastase (Parke, Davis and Company) 3 ml. pH 6.8 phosphate buffer. This mixture was then refluxed five hours with 198.7 mg. of 3-chloro-6-hydroxyacetanilide (m. p. 185°). When the reaction was cooled to 5°, solid material precipitated (m. p. 184-190°). This solid was recrystallized from 50% ethanol, 50% methanol, 50% acetic acid and then six times from 50% ethanol. From each of the last recrystallizations 0.1 ml. was plated on duplicate planchets. Compound dried from the last recrystallization (m. p. 185°) was dried over H_2SO_4 and 1.18 mg. plated on each of two planchets. The counts obtained were 15.6 and 15.2 c/m/planchet or an average of 13.1 c/m/mg.

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	13.7	13.4
5	14.9	14.4
6	6.0	10.8
7	9.4	9.5
8	4.4	2.0
9	3.8	4.1

$$\frac{13.1 \text{ c/m/mg.} \times 198.7 \text{ mg.} \times 100}{78{,}000 \text{ c/m}} = 3.34\%$$

Previously, 1.85% of the activity had been determined to be excreted as the unconjugated 3-chloro-6-hydroxyacetanilide. Therefore, 3.34% - 1.85% = 1.49%, excreted as the ethereal sulfate of 3-chloro-6-hydroxyacetanilide.

(2) 3-Chloro-6-hydroxyaniline

(a) Unconjugated 3-chloro-6-hydroxyaniline.--Three ml. radioactive urine (78,000 c/m) was refluxed five hours with 205.3 mg. of 3-chloro-6-hydroxyaniline (m. p. 139°). Solid material precipitated when the mixture was cooled. This solid was recrystallized from 25% ethanol, then six times from water and sodium hyposulfite. Planchets were plated with 0.1 ml. from each of the last four recrystallizations. Solid from the last recrystallization was plated on specific activity planchets and counts were recorded to be 1.3 and 1.9 c/m/planchet or 1.59 c/m/mg., average.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	4.4	4.5
5	0.1	0.3
6	-0.9	-0.2
7	-0.3	-0.4

$$\frac{205.3 \text{ mg.} \times 1.59 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 0.42\%,$$

unconjugated 3-chloro-6-hydroxyaniline.

(b) Glucuronide of 3-chloro-6-hydroxyaniline.--Three ml. urine from rats injected with 3-chloroacetanilide-Cl-36 was mixed with 3 ml. 1% beta-glucuronidase (Sigma) buffered at pH 6.8 with phosphate buffer. This mixture was incubated for 24 hours at 37.5°. At the end of the incubation period, 211.2 mg. 3-chloro-6-hydroxyaniline (m. p. 139°) was added and refluxed with the hydrolyzed urine for five hours. The compound was brought down by cooling and recrystallized from 50% ethanol, 25% methanol, and six times from water plus sodium hyposulfite.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	9.6	9.1
5	5.4	4.8
6	2.9	2.5
7	0.3	0.7
8	0.0	0.5

The crystalline material from the last recrystallization was plated on duplicate planchets (0.80 mg./planchet). Counts recorded were 2.5 and 4.7 c/m/planchet or an average 4.5 c/m/mg.

$$\frac{211.2 \text{ mg.} \times 4.5 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 1.22\%,$$

unconjugated and as the glucuronide of 3-chloro-6-hydroxyaniline. Therefore, 1.22% - 0.42% = 0.80%, excreted as the glucuronide of 3-chloro-6-hydroxyaniline.

(c) Ethereal sulfate of 3-chloro-6-hydroxyaniline.-- Three ml. radioactive urine from rats injected with 3-chloroacetanilide-Cl-36 was hydrolyzed with takadiastase (Parke, Davis and Company) buffered to pH 6.8 with phosphate buffer. After incubating 24 hours at 37.5° the mixture was refluxed with 199.4 mg. 3-chloro-6-hydroxyaniline (m. p. 139°). The precipitate that formed upon cooling was recrystallized from 25% ethanol, water plus sodium hyposulfite, 25% methanol, and then five times from water and sodium hyposulfite. Planchets were plated from the last five recrystallizations (0.1 ml.). Duplicate planchets were plated with material from the last recrystallization (1.08 mg./planchet) and counts recorded were 1.8 and 1.2 c/m/planchet, an average of 1.6 c/m/planchet or 1.39 c/m/mg.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	11.4	5.6
5	6.8	7.3
6	2.9	2.3
7	2.5	1.7
8	2.4	3.1

$$\frac{199.4 \text{ mg.} \times 1.39 \text{ c/m/mg.} \times 100}{78{,}000 \text{ c/m}} = 0.36\%,$$

as the unconjugated plus ethereal sulfate of 3-chloro-6-hydroxyaniline. A previous determination showed that 0.42% had been excreted as the unconjugated metabolite; therefore, none was detected as the ethereal sulfate.

e) Inorganic chloride ion

Three ml. radioactive urine (78,000 c/m) was put into a 15 ml. conical centrifuge tube and made acid by the addition of HNO_3. The urine was heated and stirred. $AgNO_3$ solution was added dropwise until precipitation of AgCl was complete. The precipitate was washed three times with distilled water, once with ethanol, and dissolved in 12 N NH_4OH. A filter stick was used to transfer the liquid into a clean test tube. Addition of HNO_3 reprecipitated AgCl and the process was repeated twice, the second time into a tared tube. The tube and AgCl were dried at 120°. The weight of AgCl was determined to be 15.5 mg. The AgCl was dissolved in 1.55 ml. 12 N NH_4OH, 0.1 ml. (1 mg.) was plated on duplicate planchets, and the activity counted. The process was repeated three times. The third and fourth specific activities were in close agreement.

Third specific activity: 100.7 and 97.4 c/m/mg.

Fourth specific activity: 102.2 and 98.7 c/m/mg.

The average of the four planchets is 99.7 c/m/mg.

$$\frac{15.5\ \text{mg.} \times 99.7\ \text{c/m/mg.} \times 100}{78{,}000\ \text{c/m}} = 1.98\%,$$

excreted as inorganic chloride ion.

3. Identification of Metabolites of 2-Chloroacetanilide

Two Sprague-Dawley rats weighing 470 and 490 g. were injected with 2-chloroacetanilide-Cl-36 (100 mg./kg. of body weight) in a 2% Methocel suspension. Each rat was placed in a metabolism cage and urine was collected as described in III, B, 1. Fifty-one ml. (sp. act. 11,260 c/m/ml.) from the first 40 hours collection was divided into 3 ml. aliquots to determine the percentage of chloride ion, the unchanged compound, the total 4- and 6-hydroxy compounds, and the following postulated metabolites of 2-chloroacetaniline:

2-chloroaniline,
2-chloro-4-hydroxyacetanilide,
glucuronide of 2-chloro-4-hydroxyacetanilide,
ethereal sulfate of 2-chloro-4-hydroxyacetanilide,
2-chloro-4-hydroxyaniline,
glucuronide of 2-chloro-4-hydroxyaniline,
ethereal sulfate of 2-chloro-4-hydroxyaniline,
2-chloro-6-hydroxyacetanilide,
glucuronide of 2-chloro-6-hydroxyacetanilide,
ethereal sulfate of 2-chloro-6-hydroxyacetanilide,

2-chloro-6-hydroxyaniline,
glucuronide of 2-chloro-6-hydroxyaniline,
ethereal sulfate of 2-chloro-6-hydroxyaniline.

a) 2-Chloroacetanilide

A 3 ml. aliquot of radioactive urine (33,780 c/m/aliquot) was used to determine the amount of unchanged 2-chloroacetanilide in the urine of rats injected with 2-chloroacetanilide-Cl-36. Non-radioactive 2-chloroacetanilide (170.4 mg.) was dissolved in acetone in a 25 ml. Erlenmeyer flask. The aliquot of radioactive urine was added and the solution was stirred at reflux temperature for 5 hours. The solution was cooled and 2-chloroacetanilide precipitated out of solution. Aqueous ethanol (50%) was used for recrystallization. The purity of the compound was established by the radioactive constancy of the recrystallizing solution.

The compound was recrystallized four times in 50% ethanol. Duplicate planchets were plated with 0.1 ml. of fluid from each recrystallization.

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
1	22.0	24.3
2	6.8	5.4
3	5.6	4.9
4	6.8	4.2

The closeness of the counts from the last three recrystallizations indicate the purity of the compound. The purified material was dried over H_2SO_4 and 5.15 mg. weighed out and diluted to one ml. in 95% ethanol. Duplicate planchets were plated with 0.2 ml. or 1.03 mg. of 2-chloroacetanilide. The counts recorded were 5.8 and 7.2 c/m/planchet, an average of 6.5 c/m/planchet or 6.3 c/m/mg.

There had been 170.4 mg. of 2-chloroacetanilide added to 3 ml. urine containing 33,780 c/m. Therefore,

$$\frac{170.4 \text{ mg.} \times 6.3 \text{ c/m/mg.} \times 100}{33{,}780 \text{ c/m}} = 3.18\%,$$

unchanged 2-chloroacetanilide.

b) 2-Chloroaniline

2-Chloroaniline (200.4 mg., b. p. 206-7°) was dissolved in 95% ethanol. Five ml. of radioactive urine containing 56,300 c/m was added to the solution and refluxed for 5 hours. Inasmuch as isolation of solid compounds from radioactive urine is simpler than isolation of liquids, the 2-chloroaniline was converted to 2-chloroacetanilide by acetylation of the amine in water. HCl was added dropwise, to form the hydrochloride, the temperature was raised to 60° and one ml. acetic anhydride was added. Three ml. saturated sodium acetate was then used to buffer the solution. Crude 2-chloroacetanilide (m. p. 82-85°) precipitated upon cooling.

The crude material was recrystallized from 50% ethanol and charcoal. Succeeding recrystallizations from 50% ethanol were made and duplicate planchets plated with 0.1 ml. from each recrystallization fluid until the c/m/planchet was constant. On the third recrystallization, the compound was white and crystalline, m. p. 87°.

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
1	not plated	
2	86.2	95.8
3	91.0	84.6
4	83.0	96.0
5	89.6	94.2

The compound was dried over H_2SO_4 and 2.45 mg. was diluted to one ml. in 95% ethanol. Duplicate planchets were plated with 0.4 ml. or 0.98 mg. of compound. Counts recorded were 64.3 and 66.4 c/m/planchet or 65.6 and 67.3 c/m/mg., an average of 66.5 c/m/mg.

The amount of 2-chloroaniline originally added to the radioactive aliquot was 200.4 mg. This is equivalent to 266.4 mg. 2-chloroacetanilide, the form to which the compound was converted. Therefore,

$$\frac{266.4 \text{ mg. x } 66.5 \text{ c/m/mg. x } 100}{56{,}300 \text{ c/m}} = 31.46\%,$$

total compound isolated as 2-chloroacetanilide.

It had been previously determined that 3.18% of the injected compound had been excreted unchanged. The amount excreted as 2-chloroaniline was, therefore, 31.46% - 3.18% = 28.28%, 2-chloroaniline in urine.

c) 4-Hydroxy Derivatives

Five ml. radioactive urine was refluxed with 5 ml. 10 N H_2SO_4 for one hour to hydrolyze the acetyl, ethereal sulfate, and glucuronide moieties. The solution was neutralized with NaOH. 2-Chloro-4-hydroxyaniline (190.2 mg.) was dissolved in acetone and refluxed for 5 hours with the hydrolyzed urine. When the mixture was cooled, Na_2SO_4 precipitated and was thoroughly washed with acetone. The excess acetone was removed from the washings and the filtrate by distillation. The compound did not precipitate until a little NaOH was added to bring the solution to about pH 8. The crude 4-chloro-2-hydroxyaniline was recrystallized from water and $Na_2S_2O_4$ to constant radioactivity.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
1	not plated	
2	310	289
3	443	461
4	441	452
5	443	448

Duplicate planchets were plated with 0.89 mg./planchet of the material that had been dried over H_2SO_4. The recorded specific activity was 118 and 123.6 c/m/planchet or 132.2 and 138.5 c/m/mg. with an average of 133.5 c/m/mg. Calculating the percentage of the total 4-hydroxy metabolites present:

$$\frac{133.5 \text{ c/m/mg.} \times 190.2 \text{ mg.} \times 100}{56{,}300 \text{ c/m}} = 45.0\%.$$

A check was made on this determination to see if further recrystallization would change this activity. Two more recrystallizations were made from water, but the filtrate was not plated. Duplicate planchets were plated with 0.91 mg./planchet of dried compound from the last recrystallization to give an activity of 121.5 and 121.9 c/m/planchet or 133.5 and 134 c/m/mg., an average of 133.7 c/m/mg.

$$\frac{133.7 \text{ c/m/mg.} \times 190.2 \text{ mg.} \times 100}{56{,}300 \text{ c/m}} = 45.1\%,$$

total 4-hydroxy metabolites present in urine.

(1) 2-Chloro-4-hydroxyacetanilide

(a) Unconjugated 2-chloro-4-hydroxyacetanilide.--Three ml. urine (33,800 c/m) from rats injected with 2-chloroacetanilide-Cl-36 was refluxed 5 hours with 174.8 mg. 2-chloro-4-hydroxyacetanilide. Precipitation of the compound occurred when the mixture was set overnight at 5°. Recrystallization from water resulted in radioactive constancy of the filtrate at the sixth and seventh recrystallizations. Duplicate planchets were plated with 0.49 mg. 2-chloro-4-hydroxyacetanilide from the last recrystallization. Average specific activity was 2.7 c/m/mg.

Recrystallization from water	c/m/planchet (duplicates)	
2	23.0	20.0
3	4.9	4.4
4	2.9	3.3
5	1.3	2.1
6	1.4	1.4
7	1.3	1.6

$$\frac{2.7 \text{ c/m/mg.} \times 174.8 \text{ mg.} \times 100}{33{,}800 \text{ c/m}} = 1.39\%,$$

unconjugated 2-chloro-4-hydroxyacetanilide.

(b) Glucuronide of 2-chloro-4-hydroxyacetanilide.--Three ml. radioactive urine (33,800 c/m) from rats injected with 2-chloroacetanilide-Cl-36 was hydrolyzed with beta-glucuronidase as described in III, B, 1, c), (1), (b). The hydrolyzed urine was refluxed five hours with 194.1 mg. 2-chloro-4-hydroxyacetanilide (m. p. 121°). The solution was set for 12 hours at 5°, and crude 2-chloro-4-hydroxyacetanilide precipitated. The material was recrystallized once in 50% ethanol, and then five times in water. Duplicate planchets were plated with 0.1 ml. of filtrate from each of the last four recrystallizations. Duplicate planchets were plated with 0.65 mg. compound from the last recrystallization. Specific activity was 8.1 and 8.4 c/m/mg.

Recrystallization from water	c/m/planchet (duplicates)	
3	9.0	8.6
4	6.2	6.2
5	8.7	8.7
6	8.4	7.4

$$\frac{8.2\ \text{c/m/mg.} \times 194.1\ \text{mg.} \times 100}{33{,}800\ \text{c/m}} = 4.71\%,$$

unconjugated plus the glucuronide of 2-chloro-4-hydroxyacetanilide. Therefore, 4.71% - 1.39% = 3.32%, identified as the glucuronide of 2-chloro-4-hydroxyacetanilide from the urine of rats injected with 2-chloroacetanilide-Cl-36.

(c) Ethereal sulfate of 2-chloro-4-hydroxyacetanilide.--Three ml. radioactive urine (33,800 c/m) was incubated with 3 ml. 1% takadiastase (Parke, Davis and Company), buffered with pH 6.8 phosphate buffer, for 24 hours at 37.5°. This mixture was refluxed with 199.7 mg. 2-chloro-4-hydroxyacetanilide (m. p. 121°) for five hours. The solid material that precipitated when the solution was cooled for 12 hours at 5° was recrystallized twice from 50% ethanol and then four times from water. Duplicate planchets were plated with 0.1 ml. of filtrate from each of the last four recrystallizations. Duplicate planchets were plated with 0.92 mg. 2-chloro-4-hydroxyacetanilide (m. p. 121°) from the last recrystallization. The recorded specific activity was 23.6 and 20.9 c/m/planchet or 25.6 and 22.7 c/m/mg., an average of 24.1 c/m/mg.

Recrystallization from water	c/m/planchet (duplicates)	
3	14.7	16.4
4	10.2	10.4
5	9.2	9.4
6	9.3	9.7

$$\frac{199.7 \text{ mg.} \times 24.1 \text{ c/m/mg.} \times 100}{33{,}800 \text{ c/m}} = 14.2\%,$$

excreted as unconjugated plus the ethereal sulfate of 2-chloro-4-hydroxyacetanilide. The amount previously determined as unconjugated was 1.39%. Therefore, 14.2% - 1.4% = 12.8%, identified as the ethereal sulfate of 2-chloro-4-hydroxyacetanilide.

(2) 2-Chloro-4-hydroxyaniline

(a) Unconjugated 2-chloro-4-hydroxyaniline.--Five ml. radioactive urine (56,300 c/m) was refluxed 5 hours with 191.2 mg. 2-chloro-4-hydroxyaniline. Successive recrystallizations from water and sodium hyposulfite caused the activity of the recrystallizing medium to drop to background levels. Duplicate planchets were then plated with 0.4 mg. 2-chloro-4-hydroxyaniline dissolved in 95% ethanol. Gross counts of 18 and 18.5 c/m/planchet were recorded. Background was 19.8 c/m. Therefore, there was no activity present as unconjugated 2-chloro-4-hydroxyaniline.

(b) Glucuronide of 2-chloro-4-hydroxyaniline.--An aliquot of urine (3 ml., 33,800 c/m) from rats injected with 2-chloroacetanilide-Cl-36 was hydrolyzed with beta-glucuronidase as described in III, B, 1, c), (1), (b). The hydrolyzed urine was refluxed five hours with 202.9 mg. 2-chloro-4-hydroxyaniline. The solution was set for 24 hours at 5°, and crude 2-chloro-4-hydroxyaniline precipitated. Recrystallization from water plus $Na_2S_2O_4$ yielded a compound of radioactive purity.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
2	7.2	6.9
3	8.1	7.4
4	3.2	3.2
5	3.1	2.9

Duplicate planchets were plated with 0.90 mg. 2-chloro-4-hydroxyaniline from the last recrystallization. Specific activity was recorded as 3.6 and 4.5 c/m/planchet or 4 and 5 c/m/mg. Calculation of the percentage of the metabolite present:

$$\frac{4.5 \text{ c/m/mg.} \times 202.9 \text{ mg.} \times 100}{33{,}800 \text{ c/m}} = 2.7\%,$$

glucuronide of 2-chloro-4-hydroxyaniline.

(c) Ethereal sulfate of 2-chloro-4-hydroxyaniline.--Three ml. radioactive urine (33,800 c/m) was incubated for

24 hours at 37.5° with 3 ml. 1% takadiastase (Parke, Davis and Company) and 3 ml. 6.8 pH phosphate buffer. The hydrolyzed urine was then refluxed 5 hours with 221.8 mg. 2-chloro-4-hydroxyaniline dissolved in acetone. Extraneous material was filtered out of the mixture. The volume of liquid was reduced by distillation before 2-chloro-4-hydroxyaniline would precipitate out of solution. Water plus sodium hyposulfite was employed to bring the material to radioactive purity.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
3	54.5	54.2
4	39.0	33.6
5	24.0	28.0
6	25.0	26.0
7	24.0	26.0

The solid from the last recrystallization was dried over H_2SO_4 (m. p. 159°). Duplicate planchets were plated with 0.84 mg. of this dried material and the specific activity recorded as 16.9 and 18.0 c/m/planchet, or 20.1 and 21.5 c/m/mg. Calculations for the percentage of the metabolite present:

$$\frac{221.8 \text{ mg.} \times 20.8 \text{ c/m/mg.} \times 100}{33{,}800 \text{ c/m}} = 13.65\%.$$

Inasmuch as no unconjugated 2-chloro-4-hydroxyaniline was detected, this figure is the amount of ethereal sulfate present in the urine of rats injected with 2-chloroacetanilide-Cl-36.

d) 6-Hydroxy Derivatives

Five ml. of radioactive urine was refluxed one hour with 5 ml. 10 N H_2SO_4 to hydrolyze the acetyl, sulfate, and glucuronide groups from the metabolites of 2-chloroacetanilide. The mixture was cooled and neutralized with NaOH. 2-Chloro-6-hydroxyaniline (186.5 mg.) was dissolved in acetone and refluxed 5 hours with the hydrolyzed material. Upon cooling, Na_2SO_4 precipitated, was washed thoroughly with acetone and the washings added to the filtrate. The excess acetone was distilled from the solution and 2-chloro-6-hydroxyaniline precipitated upon cooling (m. p. 110-115°; lit. 122°). The material was recrystallized from 50% ethanol and then to radioactive purity in water with a little sodium hyposulfite present. Duplicate planchets were plated with 0.1 ml. from each recrystallization. Seven crystallizations were necessary to get reasonably good duplication of the activity in the solvent.

Recrystallizations from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	91	100
5	69	73
6	64	67
7	65	70

The material was dried and duplicate planchets were plated with 0.46 mg. (m. p. 122°) of the compound. The specific activity of the total 6-hydroxy compounds was recorded as 12.8 and 11.2 c/m/planchet; average, 12 c/m/planchet or 25.8 c/m/mg.

Calculations of the percentage present:

$$\frac{186.5 \text{ mg.} \times 25.8 \text{ c/m/mg.} \times 100}{56{,}300 \text{ c/m}} = 8.53\%,$$

total 6-hydroxy metabolites.

(1) 2-Chloro-6-hydroxyacetanilide

(a) Unconjugated 2-chloro-6-hydroxyacetanilide.--Three ml. radioactive urine containing 27,000 c/m was refluxed 5 hours with 192.5 mg. of 2-chloro-6-hydroxyacetanilide (m. p. 190°) dissolved in acetone. The solution was cooled and the 2-chloro-6-hydroxyacetanilide that precipitated was recrystallized from 50% acetone, 50% ethanol, 25% methanol, and then twice from 50% ethanol. Duplicate planchets were plated from the last two recrystallizations; they counted just above background:

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	0.7	1.2
5	1.4	0.2

Duplicate planchets were plated with 0.97 mg. of the material from the last recrystallization (m. p. 190°). The counts recorded from the specific activity planchets: -0.4 and +0.1. Therefore, no 2-chloro-6-hydroxyacetanilide, unconjugated, was present.

(b) Glucuronide of 2-chloro-6-hydroxyacetanilide.-- Three ml. radioactive urine was added to 3 ml. 1% beta-glucuronidase, buffered to pH 6.8 with 3 ml. phosphate buffer and incubated at 37.5° for 24 hours. The mixture was then refluxed 5 hours with 205.2 mg. of 2-chloro-6-hydroxyacetanilide dissolved in acetone. Recrystallization of the solid that precipitated upon cooling was effected in 50% acetone, 50% ethanol, water, and then twice from 50% ethanol. The last two recrystallizations gave counts just above background when 0.1 ml. from each was plated on duplicate planchets.

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	1.3	1.0
5	1.0	0.4

Duplicate planchets were plated with 0.93 mg. of the material from the last recrystallization. The specific activity in c/m/mg. was 0.7 and -0.3. This averaged to be 0.2 c/m/mg. Therefore, only a negligible amount of the glucuronide of 2-chloro-6-hydroxyacetanilide was present as a metabolite of 2-chloroacetanilide.

(c) Ethereal sulfate of 2-chloro-6-hydroxyacetanilide.--Three ml. radioactive urine (27,000 c/m) was incubated with 3 ml. 1% takadiastase (Parke, Davis and Company), buffered with pH 6.8 phosphate buffer, for 24 hours at 37.5°. This mixture was then refluxed 5 hours with 176.7 mg. of 2-chloro-6-hydroxyacetanilide. The solid that precipitated when the solution cooled was recrystallized from 50% acetone, 25% ethanol, water, and then three times from 50% ethanol. Filtrate (0.1 ml.) from the last three recrystallizations was plated on duplicate planchets and revealed low but persistent activity.

Recrystallization from 50% ethanol	c/m/planchet (duplicates)	
4	1.1	0.9
5	1.5	1.0
6	0.8	1.2

Duplicate planchets were plated with 1.06 mg. material from the last recrystallization (m. p. 190°). Specific activity in c/m/mg. was recorded as 1.5 and 1.1 or an average of 1.3.

$$\frac{1.3\ \text{c/m/mg.} \times 176.7\ \text{mg.} \times 100}{27{,}000\ \text{c/m}} = 0.85\%,$$

excreted as the ethereal sulfate of 2-chloro-6-hydroxyacetanilide.

(2) 2-Chloro-6-hydroxyaniline

(a) Unconjugated 2-chloro-6-hydroxyaniline.--

2-Chloro-6-hydroxyaniline (203.2 mg.) was dissolved in acetone and refluxed 5 hours with 5 ml. radioactive urine. This compound was recovered from the mixture and twice recrystallized from 50% ethanol. The compound (m. p. 120°; lit. 122°) was recrystallized five times from water with sodium hyposulfite present to prevent oxidation of the amine. On the sixth and seventh recrystallization, the activity of the filtrate fell below background.

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
3	60.6	60.6
4	29.0	19.0
5	4.5	4.5
6	-0.4	-0.7
7	-0.1	-0.3

Dried and purified 2-chloro-6-hydroxyaniline (0.88 mg.) was plated on duplicate planchets. The counts recorded were at background: 0.2 and -0.2 c/m/planchet. Thus, no activity was detected in unconjugated 2-chloro-6-hydroxyaniline.

(b) Glucuronide of 2-chloro-6-hydroxyaniline.-- Three ml. radioactive urine (27,000 c/m) was incubated with an equal volume of 1% beta-glucuronidase and pH 6.8 phosphate buffer for 24 hours at 37.5°. The hydrolyzed urine was refluxed with 201.6 mg. of 2-chloro-6-hydroxyaniline (m. p. 122°)

dissolved in acetone. The acetone and excess water were distilled off and the solid allowed to precipitate in the cold. The material was recrystallized six times from water plus sodium hyposulfite. The product was white and crystalline (m. p. 122°). Duplicate planchets were plated from the last three recrystallizations:

Recrystallization from water + $Na_2S_2O_4$	c/m/planchet (duplicates)	
4	1.9	1.6
5	2.5	4.8
6	1.3	0.4

Specific activity planchets were plated with 0.99 mg. 2-chloro-6-hydroxyaniline and 0.8 and 0.4 c/m/planchet or an average 0.6 c/m/mg. was recorded.

$$\frac{0.6 \text{ c/m/mg.} \times 201.6 \text{ mg.} \times 100}{27{,}000 \text{ c/m}} = 0.45\%,$$

glucuronide of 2-chloro-6-hydroxyaniline present in urine as metabolite of 2-chloroacetanilide.

(c) Ethereal sulfate of 2-chloro-6-hydroxyaniline.--Three ml. radioactive urine (27,000 c/m) from rats injected with 2-chloroacetanilide-Cl-36 was incubated with an equal volume of 1% takadiastase (Parke, Davis and Company) and pH 6.8 phosphate buffer at 37.5° for 24 hours. The hydrolyzed solution was then refluxed 5 hours with 197.4 mg. of 2-chloro-6-hydroxyaniline dissolved in acetone. The excess acetone was distilled off and the precipitate obtained upon cooling was recrystallized twice from water with sodium hyposulfite present, once from 50% ethanol, and then three times from water. Duplicate planchets were plated with 0.1 ml. of filtrate from each of the last three recrystallizations:

Recrystallization from water	c/m/planchet (duplicates)	
4	6.9	5.2
5	2.2	1.7
6	2.2	3.6

Duplicate planchets were plated with 1.12 mg. of material reclaimed from the last recrystallization. The specific activity was 5.2 and 6.1 c/m/mg., an average of 5.6 c/m/mg.

$$\frac{5.6 \text{ c/m/mg.} \times 197.4 \text{ mg.} \times 100}{27{,}000 \text{ c/m}} = 4.1\%,$$

identified as the ethereal sulfate of 2-chloro-6-hydroxyaniline.

e) Inorganic chloride ion

A 3 ml. aliquot of radioactive urine (28,700 c/m) was put into a 15 ml. centrifuge tube, acidified with HNO_3, heated on a water bath, and stirred. $AgNO_3$ solution (0.1 N) was added dropwise until precipitation of AgCl was complete. The AgCl was washed three times with distilled water, once with ethanol, and then dissolved in 12 N NH_4OH. The solution was filtered by means of a filter stick into a clean tube.

The filter stick was carefully washed with ammonia water. Addition of HNO_3 reprecipitated AgCl and the entire process was repeated twice, the second time into a tared tube. The tube and contents were dried four hours at 120° and the weight of AgCl determined to be 32.6 mg. The AgCl was then dissolved in 3.26 ml. 12 N NH_4OH and two planchets plated with 0.1 ml. (1 mg.) of AgCl and the specific activity counted. The process was repeated twice on the same sample of AgCl. The second and third specific activities were constant.

Second specific activity: 5.3 and 5.7 c/m/mg.

Third specific activity: 5.5 and 5.5 c/m/mg.

$$\frac{5.5 \text{ c/m/mg.} \times 32.6 \text{ mg.} \times 100}{28{,}700 \text{ c/m}} = 0.62\%,$$

activity present as inorganic chloride ion.

C. Relative Toxicity of Acetanilide and 4-, 2-, and 3-Chloroacetanilide

The relative acute toxicities of acetanilide and 4-, 2-, and 3-chloroacetanilide were determined statistically in terms of the LD50 (27). Logarithmic-probit paper was used to plot percentage of mortality in groups of animals against the dosage administered. These graphs automatically convert mortality to probability units (probits) (67). The dosage level at the point where the curve crosses Probit 5 on the graph is taken as the LD50.

1. Toxicity in Mice

Female C57bl/6 mice were housed in wire cages, 10 mice to a cage. The cages were placed in an air-conditioned room maintained at a temperature of 22°. The animals were fed regular Purina Laboratory Chow and had access to water at all times. At the time of the experiment, the mice were 18 weeks old and each weighed 20 grams ∓ 2 grams.

Exploratory tests using 2 mice/dose were made over a wide range to determine the approximate doses to be administered. When the approximate range was established, groups of 10 mice for each of the isomeric chloroacetanilides and acetanilide were injected intraperitoneally. Each dose was given on a mg./kg. of body weight basis assuming that each mouse weighed 20 g. The dosages used were:

4-Chloro-acetanilide (mg./kg.)	3-Chloro-acetanilide (mg./kg.)	2-Chloro-acetanilide (mg./kg.)	Acetanilide (mg./kg.)
182	500	700	500
202	589	830	700
220	700	980	760
228	980	1370	830
284			980

The drug was suspended in 2% Methocel and was administered in a constant volume of 0.6 ml. Percentage of mortality was based on deaths occurring within 72 hours after administration of the drug.

2. Toxicity in Rats

Groups of female Sprague-Dawley rats (average weight 240 g.) were injected with the isomeric chloroacetanilides and acetanilide to confirm the relative toxicity of the drugs in the species used for metabolism studies. The animals were housed, 2 to a cage, in an air-conditioned room maintained at a temperature of 22°. The rats were fed regular Purina Laboratory Chow and had access to water at all times. The drugs were given intraperitoneally in a 2% Methocel suspension on a mg./kg. of body weight basis. The doses administered were:

4-Chloro-acetanilide (mg./kg.)	3-Chloro-acetanilide (mg./kg.)	2-Chloro-acetanilide (mg./kg.)	Acetanilide (mg./kg.)
237	278	700	425
246	302	760	500
255	360	830	589
278	425	980	700
284	500	1370	

Percentage of mortality was based on deaths occurring within 72 hours after administration of the drug.

IV. RESULTS AND DISCUSSION

A. Toxicity

The results of the toxicity study in the mouse with the three ring-substituted mono-chloroacetanilides and acetanilide are given in Table 1. The data from this table

TABLE 1

TOXICITY OF 4-, 3-, AND 2-CHLOROACETANILIDE AND ACETANILIDE IN C57bl/6 MICE

Compound	Dosage* (mg./kg.)	Percentage Mortality
4-Chloroacetanilide	182	0
	202	10
	220	20
	228	70
	284	100
3-Chloroacetanilide	500	10
	589	50
	700	70
	980	100
2-Chloroacetanilide	700	0
	830	40
	980	50
	1370	90
Acetanilide	500	10
	700	30
	760	50
	830	90
	980	90

* Ten animals injected at each dosage level.

were transferred to probit paper (Chart 1) and the LD50 of

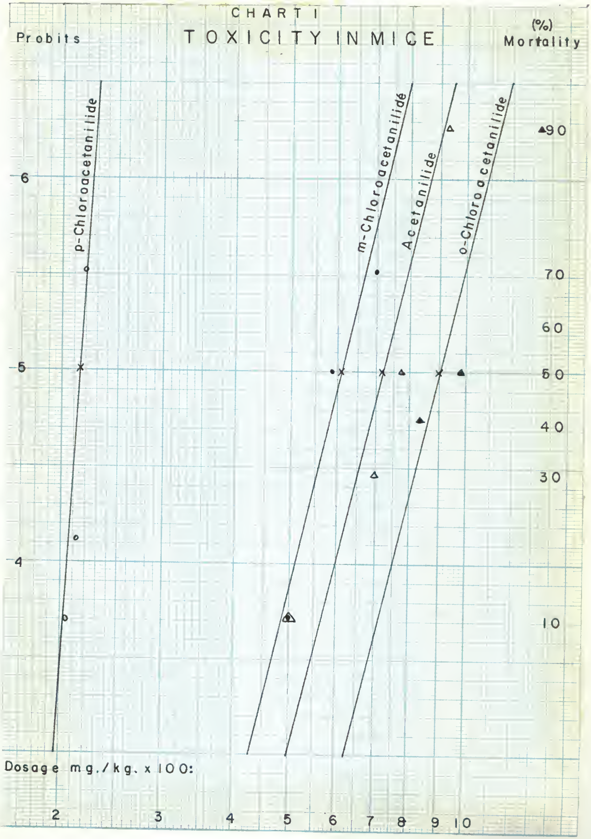


CHART I

TOXICITY IN MICE

each compound was determined by reading the dosage level at Probit 5. The LD50 of each compound expressed as mg./kg. and mM/kg. is given in Table 2. It can be seen

TABLE 2

RELATIVE TOXICITY OF 4-, 3-, AND 2-CHLOROACETANILIDE AND ACETANILIDE IN THE MOUSE (EXPRESSED AS THE LD50 OF EACH COMPOUND)

Compound	LD50 (mg./kg.)	LD50 (mM/kg.)
4-Chloroacetanilide	225	1.3
3-Chloroacetanilide	610	3.6
2-Chloroacetanilide	900	5.3
Acetanilide	715	5.3

from this table that the LD50 of 4-chloroacetanilide is much lower than for 3-, or 2-chloroacetanilide or acetanilide. When expressed on a mg./kg. basis, the toxicity of 2-chloroacetanilide is less than is that of unsubstituted acetanilide. When expressed on a mM/kg. basis the toxicity of the two drugs is the same in this study. These figures seem to indicate that introduction of the chlorine substituent, *per se*, does not increase the toxicity of the compound. The position of substitution is, however, of importance.

The results of the relative toxicity study with Sprague-Dawley rats is given in Table 3. The data from this table

TABLE 3

TOXICITY OF 4-, 3-, AND 2-CHLOROACETANILIDE AND ACETANILIDE IN SPRAGUE-DAWLEY RATS

Compound	Dosage (mg./kg.)	Percentage Mortality
4-Chloroacetanilide	237	25
	246	50
	255	75
	278*	100
	284*	100
3-Chloroacetanilide	278	0
	302	50
	360	100
	425	75
	500*	100
2-Chloroacetanilide	700	25
	760	75
	830	50
	980	75
	1370	100
Acetanilide	425	0
	500	25
	589	75
	700	100

* Two animals injected; at all other dosage levels, four animals were injected.

were transferred to probit paper (Chart 2) and the LD50 of each compound was determined by reading the dosage level at Probit 5. The results are given in Table 4. It can be seen that the relative toxicities of the isomeric chloroacetanilides and acetanilide are the same for the rat as for the mouse in the more elaborate study. 4-Chloroacetanilide is

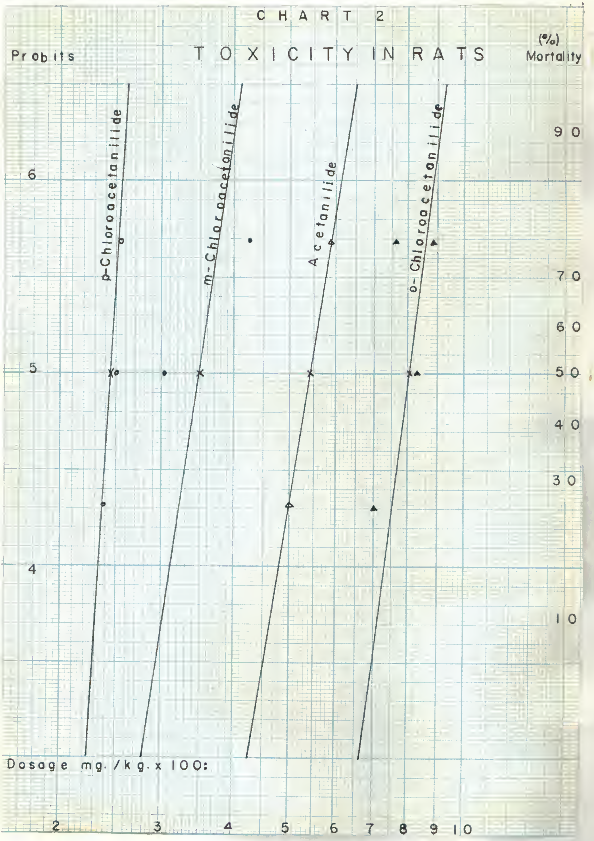


CHART 2

TOXICITY IN RATS

the most toxic drug, followed in order by 3-chloroacetanilide, acetanilide and 2-chloroacetanilide, which in this limited experiment is less toxic than acetanilide whether toxicity is expressed on a mg./kg. or a mM/kg. basis.

TABLE 4

RELATIVE TOXICITY OF 4-, 3-, AND 2-CHLOROACETANILIDE AND ACETANILIDE IN THE RAT (EXPRESSED AS THE LD50 OF EACH COMPOUND)

Compound	LD50	
	(mg./kg.)	(mM/kg.)
4-Chloroacetanilide	245	1.4
3-Chloroacetanilide	350	2.1
Acetanilide	540	4.0
2-Chloroacetanilide	810	4.7

B. Metabolism

The results of the recovery of radioactivity from the quantitative determinations of metabolites of the three ring-substituted monochloroacetanilides in male Sprague-Dawley rats are given in Table 5. The amount of unchanged compound recovered is less than 5% in all cases, and does not appear to follow the pattern of toxicity. The deacetylated compounds, 4-, 3-, or 2-chloroaniline, on the other hand, do

TABLE 5

QUANTITATIVE RECOVERY OF RADIOACTIVITY FROM THE URINE OF RATS INJECTED WITH 4-, 3-, OR 2-CHLOROACETANILIDE-CL-36 (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Item	4-Chloro-acetanilide	3-Chloro-acetanilide	2-Chloro-acetanilide
1. Unchanged chloro-acetanilide	4.63	1.34	3.18
2. Chloroanilines	2.53	8.26	28.28
3. 4-Hydroxy (para) derivatives	---	57.32	45.10
4. 2-Hydroxy (ortho) derivatives	61.96	---	---
5. 6-Hydroxy (ortho) derivatives	---	26.49	8.53
6. Total hydroxylation (Sum of Lines 3, 4, and 5)	61.96	83.81	53.63
7. Inorganic chloride ion	1.18	1.98	0.62
Total percentage recovered	70.30	95.39	85.71

follow a pattern. The most toxic compound, 4-chloroacetanilide, is the least deacetylated (2.53%); 3-chloroacetanilide is deacetylated 8.26%; while the least toxic of the trio, 2-chloroacetanilide, is deacetylated 28.28%. It is known that aniline is much more toxic than acetanilide (39). By analogy with this fact, it is not reasonable to suppose that deacetylation *per se* decreases toxicity in the chloroacetanilides. Deacetylation might increase solubility in the body fluids and, hence, permit faster elimination of the compounds from the body. This may be the case since more rapid elimination of 2-chloroacetanilide than of 4-chloroacetanilide occurs. Peak excretion of 2-chloroacetanilide occurs at 6 hours in the rat, but peak excretion is not reached until 24 hours after intraperitoneal injection of 4-chloroacetanilide. 3-Chloroacetanilide, intermediate in toxicity, has a peak excretion at 6 hours as does 2-chloroacetanilide, but traces of the drug are excreted 500 hours after administration (2).

The amount of hydroxylation determined after acid hydrolysis of the acetyl, glucuronide, and ethereal sulfate derivatives is also shown in Table 5 (lines 3, 4, 5, and 6). The 4- or para-position is, of course, blocked to hydroxylation in 4-chloroacetanilide and this inability to hydroxylate in the 4-position has been thought to be significant in increasing its toxicity. Of the two isomers with the 4-position free, however, 2-chloroacetanilide exhibits less hydroxylation in the 4-position than does the more toxic 3-chloroacetanilide. In addition, the most extensive combined ortho- and para-hydroxylation (Table 5; line 6) was also that of 3-chloroacetanilide, i.e., 83.8%. Thus, para or total hydroxylation does not appear to follow the toxic pattern. However, the hydroxylation that occurs in the 2- or 6-positions (ortho) does follow the pattern of relative toxicity. 4-Chloroacetanilide exhibits the most extensive ortho-hydroxylation (61.96%), 2.3 times as much as was found for 3-chloroacetanilide (26.49%), and 7.3 times as much as found for 2-chloroacetanilide (8.53%). The data from Table 2 show a similar order of toxicity: 4-chloroacetanilide is 2.8 times as toxic as 3-chloroacetanilide and 4.1 times as toxic as 2-chloroacetanilide.

This apparent importance of ortho-hydroxylation in the physiological response to the chloroacetanilides is analogous to its significance in the carcinogenic aromatic amines. As pointed out in the Introduction, Bonser (17) has demonstrated a relationship between the amount of injected 2-aminonaphthalene that is excreted by an animal as an ortho-hydroxy metabolite and the animal's carcinogenic response to the parent compound. Weisburger et al. (101) have demonstrated a similar phenomenon with the potent carcinogen, 2-acetylaminofluorene. These investigators found that the susceptible rat hydroxylates 2-acetylaminofluorene in the 1-, 3-, 5-, 7-, and 8-positions, but that the resistant guinea pig hydroxylates the compound only in the 7- and 8-positions and not in the 1-, 3-, and 5-positions which are the ortho positions of the carcinogen.

Table 5 also shows the results of tests made for inorganic chloride ion in the urines from rats injected with radioactive chloroacetanilides. These percentages were determined to establish whether dechlorination was carried out to any great extent in the animal body. As can be seen from the table, very little chloride ion was found.

Total recovery of the radioactivity from chloroacetanilides-Cl-36 was: 4-chloroacetanilide, 70.3%; 3-chloroacetanilide, 95.3%; 2-chloroacetanilide, 85.7%. Clearly, some unsuspected metabolites must be present in the case of the 4-isomer and possibly the 2-isomer. Aromatic amines are not known to be formers of mercapturic acids. However, halogenated compounds may be converted by the animal body to this type of metabolite. Bray _et al_. (24) found that mercapturic acid formation was a minor metabolic pathway for 2- and 4- monochloronitrobenzenes but not for 3-chloronitrobenzene. In 2- and 4-monochloronitrobenzene, mercapturic acid apparently was formed by acetylcysteyldechlorination. This pathway would not account for the entire discrepancy in the monochloroacetanilide series since only a small amount of inorganic chloride ion was found in the urine. Mercapturic acids from monochlorobenzene and polychlorobenzenes are, on the other hand, products of acetylcysteyldeprotonation (5). Possibly some metabolism of this type takes place in the chloroacetanilides. No tests were made for neutral sulfur which would have detected the presence of mercapturic acids. Neither were tests made for N-glucuronic acids nor for the sulfamic acids (20). Parke and Williams (75) have shown that in benzene some oxidation of the aromatic ring occurs. The possibility of metabolism by ring breakage was not investigated in the present study.

The results of the qualitative determinations for the individual hydroxy metabolites of 4-chloroacetanilide are given in Table 6. It can be seen from this table that the conjugated glucuronides and ethereal sulfates are found in both the acetylated and the deacetylated metabolites. Identification was also made of the unconjugated 4-chloro-2-hydroxyaniline and 4-chloro-2-hydroxyacetanilide.

The metabolites of 3-chloroacetanilide are given in Table 7. 3-Chloro-4-hydroxyacetanilide was found unconjugated, and conjugated as the glucuronide and the ethereal sulfate. A small amount of the deacetylated and unconjugated 3-chloro-4-hydroxyaniline was found to be present and 1.19% was conjugated as the ethereal sulfate, but no glucuronide

TABLE 6

QUALITATIVE RECOVERY FOLLOWING ENZYMATIC HYDROLYSES* OF HYDROXY METABOLITES FROM THE URINE OF RATS INJECTED WITH 4-CHLOROACETANILIDE-CL-36 (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Metabolite	Amount
Unconjugated 4-chloro-2-hydroxyacetanilide	2.45
Glucuronide of 4-chloro-2-hydroxyacetanilide	4.35
Ethereal sulfate of 4-chloro-2-hydroxyacetanilide	0.65
Unconjugated 4-chloro-2-hydroxyaniline	0.65
Glucuronide of 4-chloro-2-hydroxyacetanilide	0.23
Ethereal sulfate of 4-chloro-2-hydroxyaniline	1.73
Total recovery from qualitative determinations	10.06

* Recovery of 2-hydroxy metabolites following acid hydrolysis: 61.96%.

TABLE 7

QUALITATIVE RECOVERY FOLLOWING ENZYMATIC HYDROLYSES* OF HYDROXY METABOLITES FROM URINE OF RATS INJECTED WITH 3-CHLOROACETANILIDE-CL-36 (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Metabolite	Amount	
Unconjugated 3-chloro-4-hydroxyacetanilide	3.81	
Glucuronide of 3-chloro-4-hydroxyacetanilide	15.35	
Ethereal sulfate of 3-chloro-4-hydroxyacetanilide	26.43	
Unconjugated 3-chloro-4-hydroxyaniline	0.28	
Glucuronide of 3-chloro-4-hydroxyaniline	0.00	
Ethereal sulfate of 3-chloro-4-hydroxyaniline	1.19	
Total recovery of 4-hydroxy metabolites by qualitative determinations		47.06*
Unconjugated 3-chloro-6-hydroxyacetanilide	1.85	
Glucuronide of 3-chloro-6-hydroxyacetanilide	2.51	
Ethereal sulfate of 3-chloro-6-hydroxyacetanilide	1.49	
Unconjugated 3-chloro-6-hydroxyaniline	0.42	
Glucuronide of 3-chloro-6-hydroxyaniline	0.80	

TABLE 7--Continued

Metabolites	Amount
Ethereal sulfate of 3-chloro-6-hydroxyaniline	0.00
Total recovery of 6-hydroxy metabolites by qualitative determinations	7.07*

* Recovery of hydroxy metabolites following acid hydrolyses: 4-hydroxy, 57.32%; 6-hydroxy, 26.49%.

of 3-chloro-4-hydroxyaniline was identified. In the 6-hydroxy metabolites of 3-chloroacetanilide, all three of the acetylated compounds were present--the unconjugated as well as the glucuronide and the ethereal sulfate of 3-chloro-4-hydroxyacetanilide. Identification was also made of the unconjugated, deacetylated compound 3-chloro-6-hydroxyaniline. A small amount of this compound was found conjugated as the glucuronide but none of the ethereal sulfate was found.

The metabolites of 2-chloroacetanilide are listed in Table 8. In the 4-hydroxy series, unconjugated 2-chloro-4-hydroxyacetanilide was found but 2-chloro-4-hydroxyaniline was not found unconjugated. The glucuronides of acetylated and deacetylated 4-hydroxy compounds were identified, and both ethereal sulfates were present in substantial amounts. In the 6-hydroxy series of 2-chloroacetanilide metabolites, 2-chloro-6-hydroxyacetanilide was found only as the ethereal sulfate. Neither the glucuronide nor the unconjugated phenol

TABLE 8

QUALITATIVE RECOVERY FOLLOWING ENZYMATIC HYDROLYSES* OF HYDROXY METABOLITES FROM URINE OF RATS INJECTED WITH 2-CHLOROACETANILIDE-CL-36 (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Metabolite	Amount	
Unconjugated 2-chloro-4-hydroxyacetanilide	1.39	
Glucuronide of 2-chloro-4-hydroxyacetanilide	3.32	
Ethereal sulfate of 2-chloro-4-hydroxyacetanilide	12.80	
Unconjugated 2-chloro-4-hydroxyaniline	0.00	
Glucuronide of 2-chloro-4-hydroxyaniline	2.70	
Ethereal sulfate of 2-chloro-4-hydroxyaniline	13.65	
Total recovery of 4-hydroxy metabolites by qualitative determinations		33.86*
Unconjugated 2-chloro-6-hydroxyacetanilide	0.00	
Glucuronide of 2-chloro-6-hydroxyacetanilide	0.00	
Ethereal sulfate of 2-chloro-6-hydroxyacetanilide	0.85	
Unconjugated 2-chloro-6-hydroxyaniline	0.00	
Glucuronide of 2-chloro-6-hydroxyaniline	0.45	

TABLE 8--Continued

Metabolite	Amount
Ethereal sulfate of 2-chloro-6-hydroxyaniline	4.10
Total recovery of 6-hydroxy metabolites by qualitative determinations	5.40*

* Recovery of hydroxy metabolites following acid hydrolyses: 4-hydroxy, 45.1%; 6-hydroxy, 8.53%.

was identified. The deacetylated compound, 2-chloro-6-hydroxyaniline, was found in small amounts as the glucuronide (0.45%) and to a greater extent (4.1%) as the ethereal sulfate, but no unconjugated 2-chloro-6-hydroxyaniline was found.

For the qualitative determinations in Tables 6, 7, and 8, enzymatic hydrolyses were used and, as can be seen from the discrepancies between total hydroxylation and the sums of the recoveries of the individual metabolites, the determinations were not quantitative. Following enzymatic hydrolysis a mild method of isolation of the metabolites was used, i.e., stirring the base compound in solution with hydrolyzed urine for one hour at room temperature. It may well be that equilibrium of active and inactive compounds was not achieved. Sigma (82) has established that pH 6.8 is optimal for activity of beta-glucuronidase in most instances, and this was the pH at which the urines were buffered. In determinations for some glucuronides, however, it has been found that a more acid medium produces hydrolysis more efficiently (19). It is entirely feasible that a variation in conditions would produce a greater percentage of the glucuronides. Boyland *et al.* (21) has shown that some o-aminoaryl sulfates are not hydrolyzed by takadiastase. This may be the case with the ethereal sulfates of 4-chloroacetanilide derivatives. Boyland feels that this failure to hydrolyze is due to the strong basic group in the position ortho to the ester group. A contradiction to this postulate was found in the present study with the metabolites of 2-chloroacetanilide (Table 8) in which 4.1% out of a total 6-hydroxylation of 8.5% was identified as the ethereal sulfate of 2-chloro-6-hydroxyaniline.

Recovery of the individual 6-hydroxy metabolites of 3-chloroacetanilide (Table 7) was poor in comparison to the total found by acid hydrolysis. Here again it may be that the strong basic group prevented hydrolysis. No ethereal sulfate of 3-chloro-6-hydroxyaniline was found and only 1.49% of 3-chloro-6-hydroxyacetanilide which, due to acetylation, is a weaker base.

Recovery of individual 4-hydroxy metabolites was quite good from both 2- and 3-chloroacetanilide (Tables 7 and 8). In each case recovery of the ethereal sulfate was higher than that of the glucuronide. Whether this reflects the conditions as they actually exist or if hydrolysis of the glucuronide was incomplete was not determined from the present investigation.

Bray *et al.* (25) determined the extent of deacetylation of the monochloro, ring-substituted acetanilides *in vitro* as 11% for 4-chloro-, 20% for 3-chloro-, and 96% for 2-chloroacetanilide. In the present *in vivo* study the percentage of deacetylation of these compounds was: 5% for 4-chloro-, 11% for 3-chloro-, and 49% for 2-chloroacetanilide (Tables 9, 10, and 11), which is the same order found

TABLE 9

METABOLITES OF 4-CHLOROACETANILIDE-CL-36 EXCRETED ACETYLATED OR DEACETYLATED (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Item	Acetanilides	Anilines
4-Chloro-	4.63	2.53
Unconjugated 4-chloro-2-hydroxy-	2.45*	0.65*
Glucuronide of 4-chloro-2-hydroxy-	4.35*	0.23*
Ethereal sulfate of 4-chloro-2-hydroxy-	0.65*	1.73*
Total	12.08	5.14

* Values determined by qualitative methods.

TABLE 10

METABOLITES OF 3-CHLOROACETANILIDE-CL-36 EXCRETED ACETYLATED OR DEACETYLATED (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Item	Acetanilides	Anilines
3-Chloro-	1.34	8.26
Unconjugated 3-chloro-4-hydroxy-	3.81*	0.28*
Glucuronide of 3-chloro-4-hydroxy-	15.35*	0.00*
Ethereal sulfate of 3-chloro-4-hydroxy-	26.43*	1.19*
Unconjugated 3-chloro-6-hydroxy-	1.85*	0.42*
Glucuronide of 3-chloro-6-hydroxy-	2.51*	0.80*
Ethereal sulfate of 3-chloro-6-hydroxy-	1.49*	0.00*
Total	52.78	10.95

* Values determined by qualitative methods.

TABLE 11

METABOLITES OF 2-CHLOROACETANILIDE-CL-36 EXCRETED ACETYLATED OR DEACETYLATED (EXPRESSED AS PERCENTAGE OF RADIOACTIVITY IN URINE)

Item	Acetanilides	Anilines
2-Chloro-	3.18	28.28
Unconjugated 2-chloro-4-hydroxy-	1.39*	0.00*
Glucuronide of 2-chloro-4-hydroxy-	3.32*	2.70*
Ethereal sulfate of 2-chloro-4-hydroxy-	12.80*	13.65*
Unconjugated 2-chloro-6-hydroxy-	0.00*	0.00*
Glucuronide of 2-chloro-6-hydroxy-	0.00*	0.45*
Ethereal sulfate of 2-chloro-6-hydroxy-	0.85*	4.10*
Total	21.54	49.18

* Values determined by qualitative methods.

in vitro by Bray *et al*. (25). These workers were primarily interested in deacetylation *per se* and determined the hydrolysis of the acetyl group by a colorimetric test for the free amine (23). In the present study the data for the deacetylation of the hydroxylated and unhydroxylated compounds are tabulated separately. The results on the unhydroxylated compounds can be considered quantitative. The information on the percentage of deacetylation of the hydroxylated compounds, however, is incomplete. It is based on enzymatic hydrolyses of glucuronides and ethereal sulfates. This mild hydrolysis was necessary for qualitative identification but was not sufficient for quantitative recovery.

Table 11 shows the deacetylation data on 2-chloroacetanilide. This compound undergoes at least 49% deacetylation. However, 21% or more remains acetylated. Thus, in this case, the amount of acetylated compound excludes the possibility of the deacetylated metabolites of 2-chloroacetanilide reaching the 96% obtained by Bray *et al.* *in vitro*. This is probably because reacetylation is more likely to occur *in vivo* than *in vitro*.

One can conclude on the basis of the study described in this dissertation that toxicity decreases with the extent of deacetylation of the monochloro- ring-substituted acetanilides, but increases with the extent of ortho-hydroxylation.

V. SUMMARY

The relationship of physiological response to metabolism *in vivo* was investigated. This was done by relating the extent and type of detoxication of the monochloro-, ring-substituted acetanilides to their toxicity. To accomplish this the following experiments were undertaken.

1. The LD50 of 4-, 3-, and 2-chloroacetanilide was determined in C57bl/6 mice and Sprague-Dawley rats. The relative toxicity is the same in each species and the order of decreasing toxicity is: 4-, 3-, and 2-chloroacetanilide.

2. 3-Chloroacetanilide-Cl-36 was prepared in good yield based on chlorine through the chlorination of 3-nitrobenzenemercuric acetate. The 3-chloronitrobenzene was then reduced and the amine acetylated. The 4- and 2-chloro-isomers-Cl-36 had been previously prepared.

3. Postulated 2-, 4-, and 6-hydroxylated metabolites of 4-, 3-, and 2-chloroacetanilide were prepared for use as carrier compounds in inverse isotope dilution experiments.

4. Sprague-Dawley rats were injected with 100 mg./kg. of body weight of 4-, 3-, or 2-chloroacetanilide-Cl-36 and the urine from the animals was collected. Inverse isotope dilution was used to identify the following metabolites from

a) 4-chloroacetanilide:

4-chloroaniline, unconjugated 4-chloro-2-hydroxyacetanilide, the glucuronide and ethereal sulfate of 4-chloro-2-hydroxyacetanilide, unconjugated 4-chloro-2-hydroxyaniline, the glucuronide of 4-chloro-2-hydroxyaniline, and the ethereal sulfate of 4-chloro-2-hydroxyaniline.

b) 3-chloroacetanilide:

3-chloroaniline, unconjugated 3-chloro-4-hydroxyacetanilide, the glucuronide of 3-chloro-4-hydroxyacetanilide, the ethereal sulfate of 3-chloro-4-hydroxyacetanilide, unconjugated 3-chloro-4-hydroxyaniline, and the ethereal sulfate of 3-chloro-4-hydroxyaniline; unconjugated 3-chloro-6-hydroxyacetanilide, the glucuronide of 3-chloro-6-hydroxyacetanilide, unconjugated 3-chloro-6-hydroxyaniline, and the glucuronide of 3-chloro-6-hydroxyaniline.

c) 2-chloroacetanilide:

2-chloroaniline, unconjugated 2-chloro-4-hydroxyacetanilide, the glucuronide of 2-chloro-4-hydroxyacetanilide, the ethereal sulfate of 2-chloro-4-hydroxyacetanilide, the glucuronide of 2-chloro-4-hydroxyaniline, and the ethereal sulfate of 2-chloro-4-hydroxyaniline; the ethereal sulfate of 2-chloro-6-hydroxyacetanilide, the glucuronide of 2-chloro-6-hydroxyaniline, and the ethereal sulfate of 2-chloro-6-hydroxyaniline.

5. A quantitative determination of ortho- and para-hydroxy derivatives of the three chloroacetanilides was made following acid hydrolyses of the urines. It was found that neither total nor para-hydroxylation followed the order of toxicity of the chloroacetanilides in rats and mice, but the extent of ortho-hydroxylation was in the same order as was the toxicity of the chloroacetanilides, i.e., 4-chloroacetanilide is the most toxic and undergoes the greatest amount of hydroxylation in the ortho-position; 3-chloroacetanilide is intermediate in toxicity and extent of ortho-hydroxylation; 2-chloroacetanilide is the least toxic and exhibits the least ortho-hydroxylation.

6. Quantitative determinations were made for deacetylation of non-hydroxylated metabolites and qualitative determinations were made for deacetylation of hydroxylated metabolites. A decrease in toxicity was noted with an increase in deacetylation of the compounds: 4-chloroacetanilide was the least deacetylated and most toxic; 3-chloroacetanilide, intermediate in deacetylation and toxicity; 2-chloroacetanilide, the most extensively deacetylated and the least toxic.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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